NEUROTROPHIN SIGNALING IS REQUIRED FOR GLUCOSE INDUCED INSULIN SECRETION

by
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ABSTRACT

Insulin secretion by pancreatic islet β-cells is critical for glucose homeostasis, and a blunted β-cell secretory response is an early deficit in type 2 diabetes. While considerable attention has focused on mechanisms controlling β-cell mass, less is known about factors governing β-cell secretory responses. Here, we uncover a regulatory mechanism by which glucose recruits vascular-derived neurotrophins to control insulin secretion. Nerve Growth Factor (NGF), a classical trophic factor for nerve cells, is expressed in pancreatic vasculature while its TrkA receptor is localized to islet β-cells. High glucose rapidly enhances NGF secretion and TrkA phosphorylation in islets. Vascular-specific deletion of NGF, pancreas-specific *TrkA* deletion, or acute disruption of TrkA activity impairs glucose tolerance and insulin secretion in mice. Furthermore, we show that high glucose activates TrkA and NGF potentiates insulin secretion in human islets, and these effects are blunted in islets from pre-diabetic individuals. We find that internalized TrkA receptors promote insulin granule exocytosis via F-actin reorganization. These results reveal a non-neuronal role for neurotrophins, and identify a new regulatory pathway in insulin secretion that can be targeted to ameliorate β-cell dysfunction.

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PREFACE

Science is a collaborative field that relies on the work of many people to accomplish a common goal. I am truly fortunate to have had outstanding support and mentorship from many people in the course of my graduate career. First, I need to acknowledge my mentor, Rejji Kuruvilla, to whom I am incredibly thankful for all of her training and encouragement. Rejji is unique among PhD mentors in her level of dedication to her students and their projects. I am continually astounded at the breadth of her knowledge and the eloquence with which she organizes information into writing and presentations. Working with Rejji has taught me how to more effectively organize and present my findings. Additionally, our conversations have led me to streamline my efforts in lab and efficiently design relevant experiments to approach scientific problems. Finally, Rejji's level of tenacity has been the driving force behind a number of experiments in this work. She truly puts meaning to the phrase "Where there's a will, there's a way." Rejji can always think of something to try to either move a study forward or make a finding stronger. In the future, I hope to be able to bring a similar level of intensity and drive to my own projects.

Working on a topic that diverges from the primary focus of the lab can be quite challenging, and I was extremely privileged to have the guidance of a previous graduate student, Philip Borden, when I joined the lab. Phil patiently taught me techniques that have been essential to my project such as glucose tolerance tests and islet isolations. His dedication to detail and thoroughness in teaching have been instrumental in my success at the bench and I am forever thankful for all of his help.

The environment that one works in can have a great influence on productivity. I would like to thank the numerous other members of the lab, both past and present, for creating such an enjoyable place to walk into every day. The level of support and understanding between lab members truly makes them feel like family. Another significant feature of my training in graduate school has been being part of the Mouse Tri-Lab. The Tri-Lab is an amazing group of scientists. I've learned more than I can say through participation in Tri-Lab meetings, practice talks, and journal clubs. The intellectual discussion and presentation of a diverse set of projects has made me a better rounded scientist than I ever could have achieved on my own.

I must also thank my thesis committee, Samer Hattar, Seth Blackshaw, Stephen Leach, and Mehboob Hussain, as well as my reader, Robert Johnston, for all their generosity with time, reagents, and advice. Having intellectual input from such a diverse and strong group of scientists has made this work stronger and challenged the way I think about my science. To Mehboob Hussain, I am additionally grateful for the time he took during a busy conference to introduce me to members of the islet biology field, and for many meetings to provide technical expertise and discuss the implications of my findings.

It may go without saying that graduate school is not easy. The personal investments of time, energy, and emotion often make for a rocky journey. I have been incredibly fortunate to have the support of many people, including family, friends, and colleagues, as I strived to complete this body of work. With a classically trained pulmonary physiologist for a mother (Pamela Houtz), a plant biochemist for a father (Robert Houtz), and an entomologist for a brother (Philip Houtz), I have always been able to take comfort in a family of scientists who fully understand the significance of submitting a manuscript or

finishing a key set of experiments. Beyond their scientific expertise, they have been ever supportive in helping me with challenges outside of the lab, be it the physical endeavor of moving into an apartment without AC in the middle of July or emotional support on the phone late at night. I have also been extremely fortunate to have the loving support of my boyfriend, Bradly Tinney. Brad has helped with everything from making meals and feeding my cats, to diligently sitting with Rejji and me in her office late on a Friday evening in order to find and fix elusive wing-dings in our manuscript. Words cannot adequately express how grateful I am to everyone who has encouraged and supported me for the past 6 years of my graduate career.

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LIST OF ABBREVIATIONS

6-OHDA	=	6-hydroxydopamine
ADP	=	Adenosine Diphosophate
ATP	=	Adenosine Triphosphate
AUC	=	Area Under the Curve
BDNF	=	Brain Derived Neurotrophic Factor
β-gal	=	Beta-galactosidase
cAMP	=	Cyclic Adenosine Monophosphate
Cdc42	=	Cell Division Control Protein 42
CGRP	=	Calcitonin gene-related peptide
CIPA	=	Congenital Insensitivity to Pain with Anhidrosis
CNTF	=	Cilliary Neurotrophic Factor
CREB	=	cAMP Response Element Binding Protein
CsA	=	Cyclosporin
DAG	=	Diacylglycerol
DBA	=	Dolichos Biflorus Agglutinin
EEA1	=	Early Endosome Antigen 1
EM	=	Electron Microscopy
Epac2	=	Exchange protein directly activated by cAMP 2
ER	=	Endoplasmic Reticulum
ERK	=	Extracellular Signal Related Kinase
F-actin	=	Filamentous actin
FAK	=	Focal Adhesion Kinase
GABA	=	Gamma-Aminobutyric Acid
G-actin	=	globular actin
GIP	=	Gastric inhibitory polypeptide
GLP-1	=	Glucagon-like peptide-1
GPCR	=	G-protein-coupled receptor
GSIS	=	Glucose Stimulated Insulin Secretion
GSK-3β	=	Glycogen synthase kinase 3
GTP	=	Guanosine triphosphate
HBSS	=	Hank's Balanced Salt Solution
i.p.	=	intraperitoneal
IIDP	=	Integrated Islet Distribution Program
IL-6	=	Interleukin 6
KATP	=	ATP-sensitive potassium channel
KCl	=	potassium chloride
KRHB	=	Krebs-Ringer HEPES buffered
MAPK	=	Mitogen-activated proetin kinase
MLCK	=	Myosin Light-Chain Kinase

MIP	=	Mouse Insulin Promoter
Myh11	=	Myosin heavy-chain 11 (SMA)
NGF	=	Nerve Growth Factor
NT-3	=	Neurotrophin-3
NWASP	=	N-Wiskott-Aldrich Syndrom Protein
PACAP	=	Pituitary adenylate cyclase-activating peptide
PAK1	=	P21 Protein-Activated Kinase 1
PBS	=	Phosphate Buffered Saline
PDGFRβ	=	Platelet -derived growth factor receptor
Pdx1	=	Pancreatic and duodenal homeobox 1
PECAM	=	Platelet endothelial cell adhesion molecule
PFA	=	paraformaldehyde
PI3K	=	Phosphoinositide 3-kinase
PIP2	=	Phosphatidylinositol bisphosphate
PLC	=	Phospholipase C
qPCR	=	quantitative polymerase chain reaction
Rac1	=	Ras-related C3 botulinum toxin substrate 1
Rap1	=	Ras-related protein 1
Robo	=	roundabout
SEM	=	standard error of the mean
SMA	=	smooth muscle actin
SNAP	=	synaptosomal-associated protein
SNARE	=	Soluble NSF Attachement Protein Receptor
SRF	=	Serum Response Factor
TH	=	Tyrosine Hydroxylase
TIRF	=	Total Internal Reflection Fluorescence
Tmxfn	=	Tamoxifen
TNF	=	Tumor Necrosis Factor
Trk	=	Tropomyosin receptor kinase
VAMP	=	Vesicle-associated membrane protein
VEGF	=	Vascular endotheilial growth factor
VGCC	=	Voltage-gated calcium channel
VMAT2	=	Vesicular Monoamine Transporter 2
WT	=	wildtype
YFP	=	yellow fluorescent protein

INTRODUCTION

This chapter was previously submitted for publication at Developmental Cell:

Jessica Houtz, Philip Borden, Alexis Ceasrine, and Rejji Kuruvilla (2016). *Neurotrophin signaling is required for glucose-induced insulin secretion.*

Insulin-producing β-cells are a small population of endocrine cells that comprise less than 2% of the pancreas, yet their function is imperative to the maintenance of blood glucose homeostasis. In response to elevated blood glucose levels, β-cells secrete the hormone insulin, which triggers glucose uptake by peripheral tissues. Blunted insulin secretion by β-cells is one of the earliest features of type 2 diabetes, a disease affecting almost 300 million people worldwide (Guariguata et al., 2014), observed even in pre-diabetic individuals and thought to precede the onset of overt hyperglycemia (Weir and Bonner-Weir, 2004). Strikingly, it has been postulated that by the time a diagnosis of diabetes is made, patients have lost almost 80% of β-cell function (Defronzo, 2009). Although the loss of β-cell secretory responses precedes a reduction in β-cell mass during the development of type 2 diabetes (Defronzo, 2009, Weir and Bonner-Weir, 2004), research has predominantly focused on mechanisms governing β-cell proliferation (Vetere et al., 2014, Wang et al., 2015, Stewart et al., 2015).

While glucose is the primary stimulus for insulin secretion, the β-cell secretory response is potentiated by extrinsic signals that include fatty acids and amino acids, peptide hormones, and neurotransmitters that play essential roles in the process (Henquin et al., 2003, Prentki et al., 2013, Rorsman and Braun, 2013, Caicedo, 2013, Campbell and Drucker, 2013). Glucose-stimulated insulin secretion (GSIS) involves the key steps of; (1) glucose uptake in β-cells, (2) mitochondrial metabolism to alter the ATP/ADP ratio, (3) closure of ATP-sensitive K^+-channels and subsequent β-cell plasma-membrane depolarization, (4) the opening of voltage-gated Ca^{2+}-channels, and (5) Ca^{2+}-dependent exocytosis of insulin granules (MacDonald et al., 2005). Extrinsic signals influence GSIS either by producing metabolic intermediates within β-cells, or by generating signaling

second messengers that impinge on β-cell electrical activity and/or insulin exocytosis (Prentki et al., 2013, Rorsman and Braun, 2013). Because the nutrient, hormonal, and neural inputs are precisely regulated by glucose itself, together they ensure that insulin secretion remains glucose-dependent at much lower plasma glucose concentrations than are effective *in vitro* (Henquin et al., 2003). Thus, a small post-prandial increase in plasma glucose elicits a larger insulin response than predicted from *in vitro* dose-response curves, due to interactions between glucose and non-glucose stimuli. Conversely, the exquisite glucose-dependence of these non-glucose insulin secretagogues is also a necessary protection against hypoglycemia (Henquin et al., 2003).

Neurotrophins are soluble peptide factors known predominantly for their functions in neuronal survival, axon growth, and synaptic communication in the vertebrate nervous system (Huang and Reichardt, 2001a). Although neurotrophins and their Trk receptor tyrosine kinases are expressed in non-neuronal tissues including the pancreas (Tessarollo, 1998), little is known about their *in vivo* functions outside of the nervous system. Based on cell culture studies, autocrine signaling by the classical neurotrophin, Nerve Growth Factor (NGF), has been implicated in β-cell survival and secretory function (Rosenbaum et al., 2001, Navarro-Tableros et al., 2004). However, physiological roles for neurotrophin signaling within pancreatic endocrine cells remain undefined. The need to understand the physiological relevance of neurotrophins in islet function is underscored by evidence in humans that altered neurotrophin secretion and/or signaling could contribute to the etiology of diabetes (Pittenger and Vinik, 2003, Schreiber et al., 2005, Bullo et al., 2007, Kim et al., 2009). In humans, mutations in the *TrkA* gene encoding for the NGF receptor, cause a form of hereditary peripheral neuropathy called congenital insensitivity to pain and anhidrosis

(CIPA) (Indo et al., 1996). Children with CIPA show decreased insulin secretion in response to a glucose challenge, suggesting that NGF signaling may play a role in insulin responses in humans (Schreiber et al., 2005). Furthermore, altered circulating NGF levels have been noted in type 2 diabetes (Bullo et al., 2007, Kim et al., 2009), although whether this reflects a cause or effect in disease pathogenesis remains undefined.

Here, we uncover a fundamental role for neurotrophin signaling in controlling glucose-stimulated insulin secretion, and elucidate the cellular underpinnings. We found that NGF is robustly expressed in pancreatic vascular contractile cells, whereas its TrkA receptor is localized to islet β-cells. Elevated glucose rapidly increased NGF secretion and stimulated TrkA phosphorylation in isolated islets. Pancreas-specific *TrkA* deletion as well as acutely inactivating TrkA signaling, using a chemical-genetic approach, impaired glucose tolerance and reduced insulin secretion in mice. TrkA activity promotes insulin granule localization to the β-cell plasma membrane via disassembly of a rigid F-actin barrier. Furthermore, Trk-mediated endosomal signaling, a critical determinant of neurotrophin actions in neurons, is conserved and functionally important in pancreatic β-cells, since endocytosis-deficient TrkA receptors were incapable of mediating actin remodeling and insulin secretion. These findings elucidate a new pathway by which glucose promotes Trk-mediated actin reorganization to trigger insulin secretion in β-cells.

CHAPTER 1: EXPRESSION OF NGF AND TRKA IN THE PANCREAS

Portions of this chapter were previously submitted for publication at Developmental Cell: Jessica Houtz, Philip Borden, Alexis Ceasrine, and Rejji Kuruvilla (2016). *Neurotrophin signaling is required for glucose-induced insulin secretion.*

INTRODUCTION

The presence of neurotrophins and their cognate Trk receptors in the pancreas has been well established for several decades, (Kanaka-Gantenbein et al., 1995b, Miralles et al., 1998, Shibayama and Koizumi, 1996) however, their precise cellular localization within this complex organ has remained elusive due to notoriously unreliable antibodies for immunostaining and lack of expression reporter systems. Given the multitude of cell types found in the pancreas: acinar and ductal cells, five different endocrine cell types, vascular cells, enteric neurons and innervating fibers of the sensory and autonomic nervous system, glial cells, and immune cells, identifying the sources of both ligand and receptor are important for determining how neurotrophin signaling occurs in this organ. Previous studies have suggested that insulin-secreting β-cells express NGF that functions in an autocrine manner (Vidaltamayo et al., 2003, Rosenbaum et al., 1998). However, these experiments were limited to *in vitro* analyses of dissociated islets, and expression of NGF could therefore be an artifact of cell culture conditions.

Early studies performed in the developing rat pancreas also indicate that all pancreatic cell types express TrkA at embryonic day 12; however, by adulthood, this expression is reduced and restricted to endocrine cells (Miralles et al., 1998). Despite these findings, there is contention in the literature regarding the identity of the pancreatic and specifically the endocrine cell types that continue to express TrkA in the adult (Teitelman et al., 1998). Additional studies have investigated the expression of other neurotrophin receptors in the pancreas and found that glucagon-secreting alpha cells express the high affinity BDNF receptor, TrkB (Hanyu et al., 2003). In this study, we use a combination of

ligand binding and an NGF reporter mouse to definitively show which pancreatic cell types express TrkA receptors and the source of the NGF ligand.

RESULTS

We first confirmed expression of TrkA and NGF in both the embryonic pancreas and adult islets using RT-PCR. While levels of NGF and TrkA appear to be reduced in adult islets compared to embryonic pancreas, samples from adult islets are enriched for endocrine cells at the expense of other cell types including ductal cells and vasculature **(Fig. 1-1)**.

It has long been held that NGF is expressed in limiting quantities by tissues that are innervated by the peripheral nervous system (Cohen and Levi-Montalcini, 1956, Deppmann et al., 2008). β-cells are richly innervated by the autonomic nervous system (Rodriguez-Diaz et al., 2011), and have been reported to express NGF by RT PCR and western blot analysis (Pierucci et al., 2001, Rosenbaum et al., 1998). We find that $NGF^{LacZ/+}$ reporter mice show strong X-gal staining in vascular structures **(Fig. 1-2A)**. Additional immunohistochemical analysis with antibodies against SMA and PDGFRβ show that NGF is primarily expressed in smooth muscle cells and intra-islet pericytes respectively **(Fig. 1-2B,C)**. No reporter expression is detected in pancreatic ducts or endothelial cells of the intra-islet vasculature **(Fig. 1-3A,B)** These results do not rule out any production of NGF by β-cells as previously reported (Rosenbaum et al., 1998), and indeed we see beta-galactosidase activity in dissociated β-cells from $NGF^{LacZ/+}$ mice in culture **(Fig. 1-3C,D)**. In dissociated islet cultures, we found most of the NGF labeling to be in intra-islet pericytes **(Fig. 1-3E and 1-3E')**. Together, these results suggest that vascular contractile cells are the likely source of NGF in the pancreas.

In order to determine which cell types in the pancreas express NGF receptors, we performed ligand binding with biotinylated NGF (200ng/mL). I found the highest concentration of binding occurred within islets, and colocalized with insulin **(Fig 1-4A)**. Although NGF binds with high affinity to TrkA receptors, β-cells are also known to express the low affinity neurotrophin receptor, p75 which could contribute to the observed NGF ligand-binding signal in the pancreas (Pierucci et al., 2001, Raile et al., 2006). However, immunostaining for p75 reveals distinctly neuronal expression of p75 with no significant signal observable within islets **(Fig. 1-4B)**, indicating that the primary source of TrkA expression in the adult pancreas is β-cells.

Figure 1-1: NGF and TrkA expression in the pancreas

RT-PCR analysis of NGF and TrkA expression in E15 pancreas and islets isolated from 1 month old wildtype mice. NGF and TrkA expression appear to be reduced in adult islets compared to E15 pancreas.

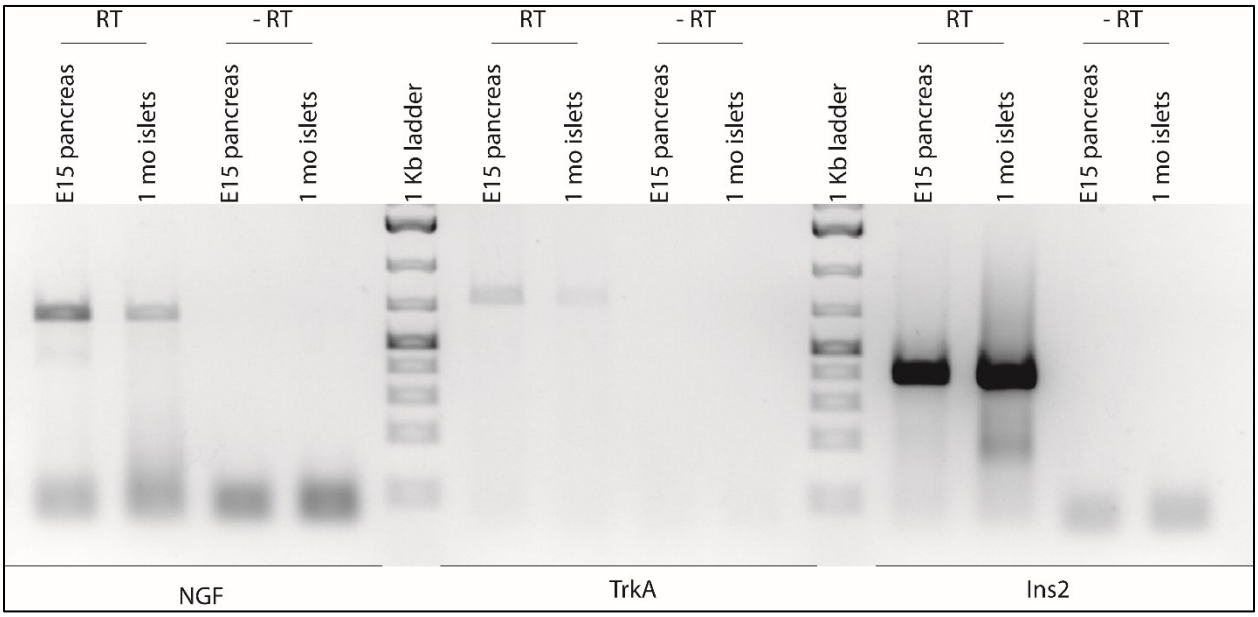


Figure 1-2: NGF is expressed in pancreatic vascular contractile cells

(A) X-gal staining reports prominent NGF expression in large diameter blood vessels (red arrows) outside islets, and also within islets (red arrowheads) in tissue sections from $NGF^{LacZ/+}$ mice. An islet is outlined in dashed lines, and shown in higher magnification in the right panel. Scale bar, 100 μm **(B,C)** Immunostaining shows co-localization of β-galactosidase (red) with smooth muscle actin (SMA, green) in large blood vessels, and with PDGFRβ (green), a pericyte marker, in the intra-islet microvasculature. Insulin staining is shown in blue. **(C')** shows a higher magnification image of **(C)**. Scale bars, 100 μm for **(C)** and 50 μm for **(C')**. Representative images in **(A-C)** are shown from five $NGF^{LacZ/+}$ mice.

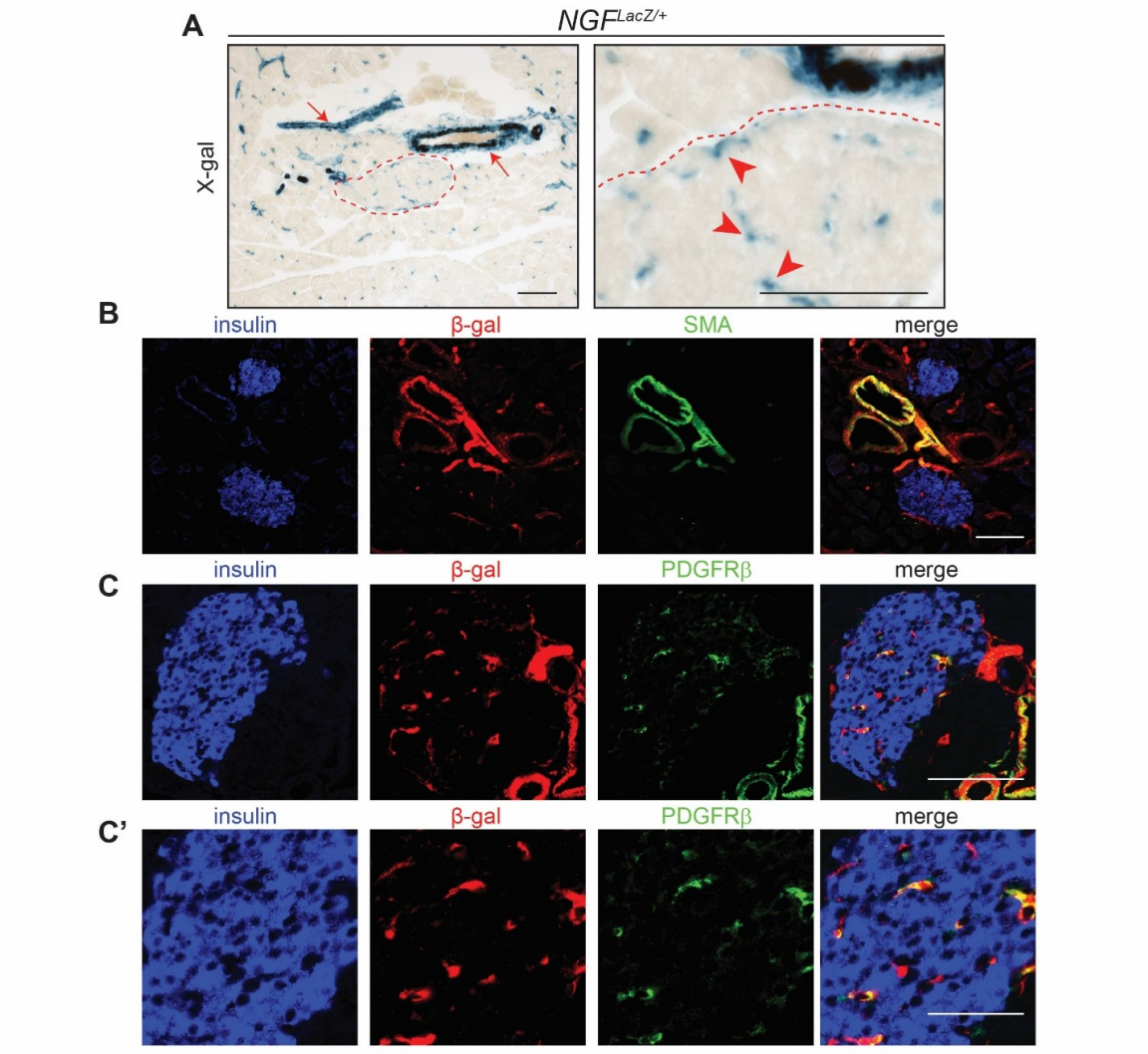

A
NGF LacZ/+
X-gal
B
insulin
β-gal
SMA
merge
C
insulin
β-gal
PDGFRβ
merge
C'
insulin
β-gal
PDGFRβ
merge

Figure 1-3: NGF is undetectable in endothelial and ductal cells, and lowly expressed in cultured β-cells

(A,B) Immunostaining for β-galactosidase that reports NGF expression shows little co-localization with endothelial cells, stained with PECAM, or pancreatic ducts, labeled with DBA, in tissue sections from $NGF^{LacZ/+}$ mice. Islets are marked by insulin immunostaining. Scale bar, 50 μm. **(C,D)** Single dissociated $NGF^{LacZ/+}$ β-cells in culture show low expression of β-galactosidase compared with control $NGF^{+/+}$ β-cells. β-cells were identified by co-labeling with insulin. Representative images are shown from at least 3 animals per genotype that were analyzed. Scale bar, 5 μm. **(E)** In dissociated islet cultures, β-galactosidase immunostaining shows NGF expression predominantly in pericytes. Arrowheads indicate a few isolated β- cells NGF expression. The pericytes and β-cells are marked by PDGFRβ and insulin immunostaining respectively. **(E')** shows a higher magnification of boxed region in (e). Scale bars, 50 μm for **(E)** and 20 μm for **(E')**.

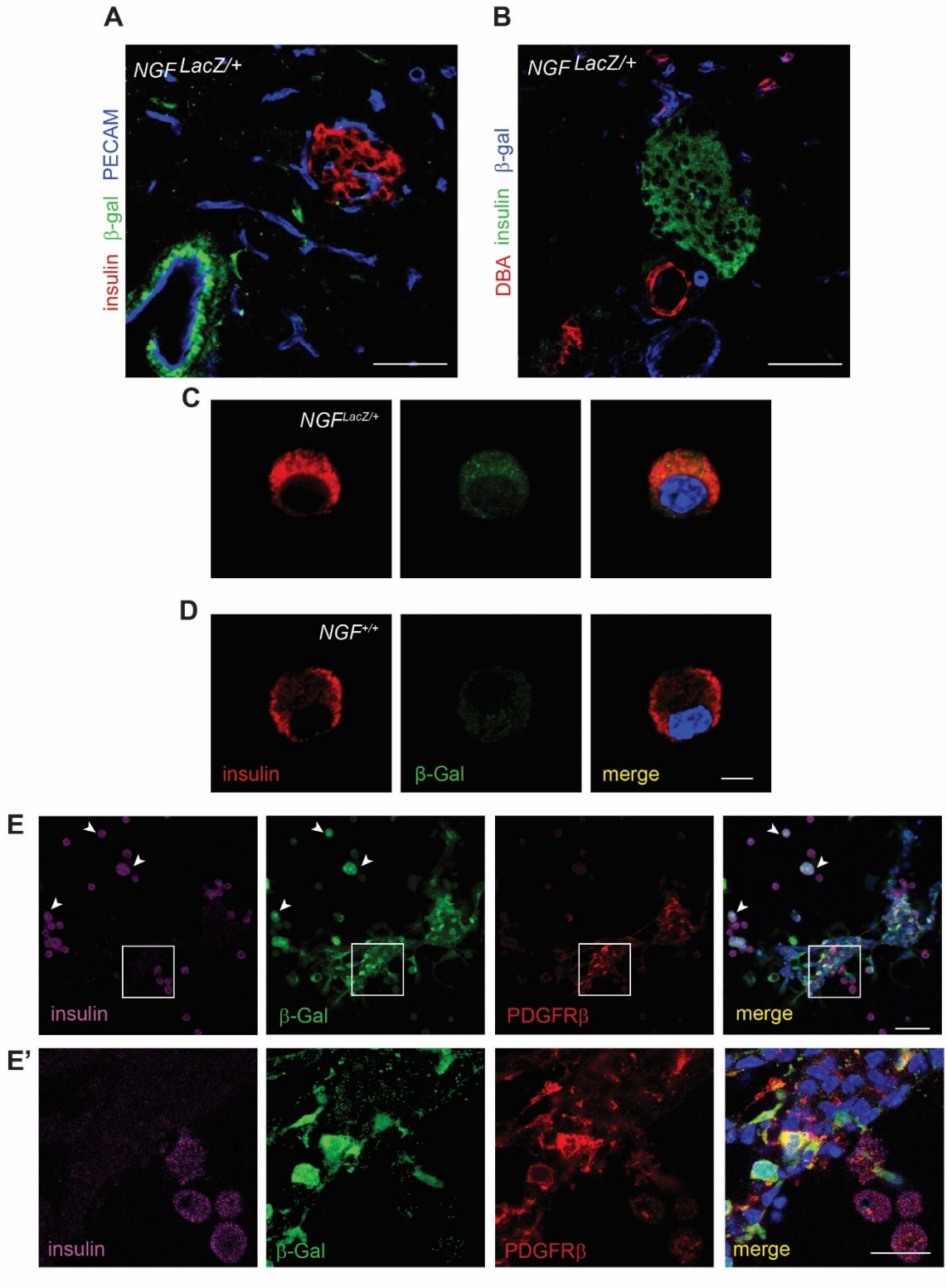
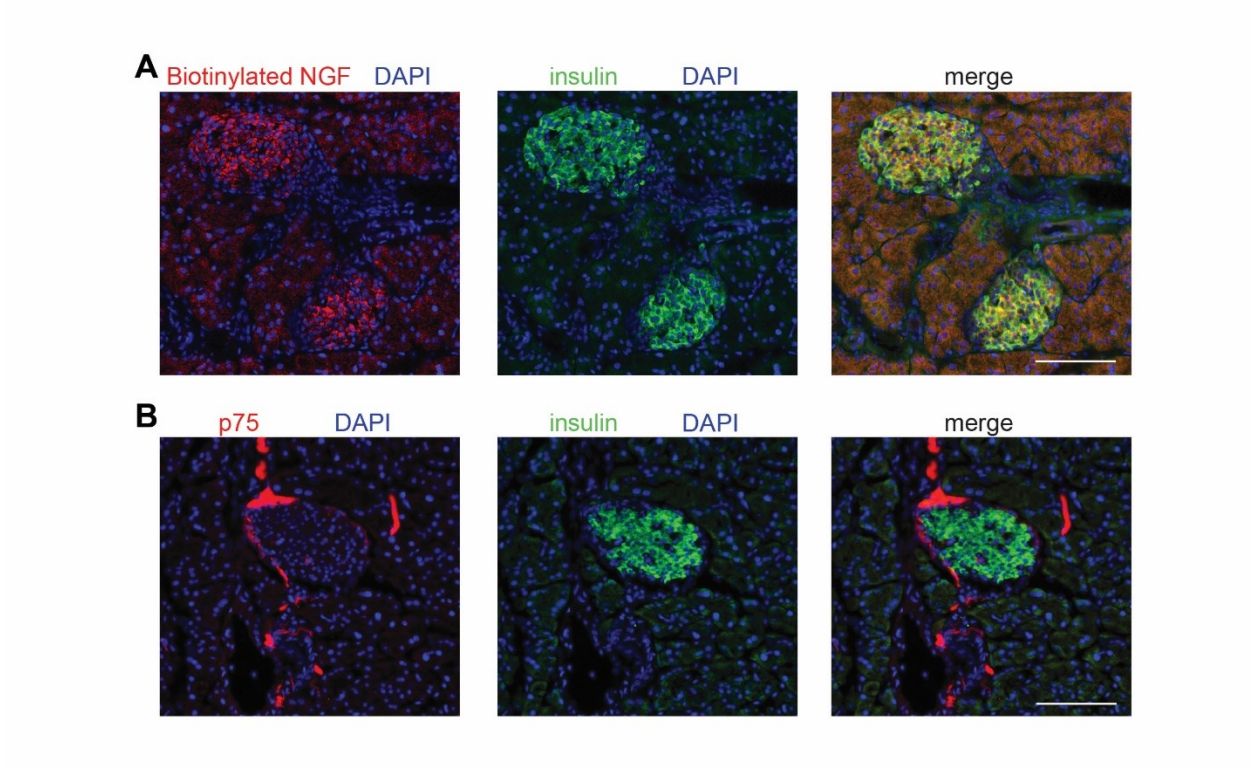

A
NGF LacZ/+
insulin β-gal PECAM
B
NGF LacZ/+
DBA insulin β-gal
C
NGFLacZ/+
D
NGF+/+
insulin
β-Gal
merge
E
insulin
β-Gal
PDGFRβ
merge
E'
insulin
β-Gal
PDGFRβ
merge

Figure 1-4: Neurotrophin receptors are expressed in the adult pancreas

(A) Biotinylated NGF binding is detected in insulin-positive β-cells in pancreatic tissue sections. **(B)** p75 immunostaining shows expression in fibers innervating the islet perimeter, but not within islets. Scale bar, 100 µm



DISCUSSION

Islets are a major target of sympathetic innervation in the pancreas, and there are several lines of evidence to support the belief that β-cells provide NGF to innervating nerves (Rosenbaum et al., 1998, Vidaltamayo et al., 2003). This led to a model where neurotrophin signaling in islets occurs in an autocrine fashion, in which β-cells secrete NGF to activate TrkA receptors on the cell surface. In contrast to this view, we have found that pancreatic contractile cells of the pancreatic vasculature predominantly contribute to NGF expression in the pancreas. Smooth muscle cells that ensheath large diameter blood vessels express the highest levels of NGF broadly across the pancreas, while intra-islet pericytes contribute to local NGF levels within islets. Loss of endothelial cells in the adult pancreas has little influence on islet function or glucose tolerance(Reinert et al., 2013), but there has been no thorough investigation of the role of other vascular cell types such as smooth muscle cells and pericytes play in islet biology. Our observations of the spatial pattern of NGF and TrkA expression in the pancreas are most consistent with a paracrine effect of vascular-derived NGF on neighboring β-cells.

Using biotinylated NGF, we were able to detect binding in sections of adult pancreas, specifically within β-cells in the islets of Langerhans. These results agree with previous findings indicating that TrkA expression in the pancreas is restricted to islets in adulthood. However, other studies suggest TrkA expression in several exocrine pancreatic cell types during development (Miralles et al., 1998, Kanaka-Gantenbein et al., 1995a). Thus, a comprehensive time-course analyzing TrkA expression in the pancreas would

significantly augment our current understanding of when and where TrkA signaling is relevant in islets.

METHODS

Mice

All procedures relating to animal care and treatment conformed to Johns Hopkins University Animal Care and Use Committee (ACUC) and NIH guidelines. Mice were housed in a standard 12:12 light-dark cycle. Mice were maintained on a *C57BL/6* background, or mixed *C57BL/6* and *129P*, or *C57BL/6* and *FVB* backgrounds. Both sexes were used for analyses at 1-2 months of age, unless stated otherwise in the figure legends. $NGF^{LacZ/+}$ mice were a generous gift from Dr. David Ginty (Harvard).

Antibodies

Primary antibodies for immunofluorescence include guinea pig anti-insulin (1:300, abcam ab7842), rabbit anti-p75 (1:200, Promega G323A), chicken anti-beta-galactosidase (1:100, Millipore AB3403-I), rat anti-PECAM (CD31) (1:200, BD Biosciences 550274), FITC-mouse-anti-SMA (1:500, Sigma F3777), and rabbit anti-PDGFRβ (1:100, abcam ab32570). Fluorescent secondary antibodies were Alexafluor-350, -488,-546, or -647-conjugated and used at 1:200 (Invitrogen)

X-gal staining, immunohistochemistry, and islet morphology

Pancreatic NGF receptors were labeled by incubating 10μm thick mouse tissue sections with biotinylated NGF (200 ng/ml) for 1 hour at room temperature. Biotinylated NGF was prepared according to the manufacturer's instructions using EZ-Link™ Micro Sulfo-NHS-Biotinylation kit (ThermoFisher Scientific, 21925). Following incubation with biotinylated NGF, tissue sections were washed and immunostained for insulin as described below.

Biotinylated NGF was detected using Alexa-546-conjugated streptavidin (1:200, Invitrogen, S11225). Sections were imaged using a Zeiss AxioImager M1 microscope equipped with an Axiocam HRc camera.

For X-gal staining, pancreata from $NGF^{LacZ/+}$ mice were fixed in 0.2% glutaraldehyde and 2mM $MgCl_2$ in PBS overnight at 4ºC. Tissues were equilibrated in 30% sucrose and embedded in OCT. 10 µm-thick sections were collected and stained with a solution containing X-gal (1 mg/ml, Invitrogen), 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, 2mM $MgCl_2$, 0.1M NaPO4, 0.01% sodium deoxycholate, and 0.02% NP40.

For immunofluorescence, tissues were fixed overnight in 4% paraformaldehyde (PFA) (Sigma) at 4ºC, cryoprotected in 30% sucrose overnight, frozen in OCT, and 10µm thick sections were collected. Sections were permeabilized in PBS with 1% Triton X-100, blocked in 5% goat serum with 0.1% Triton X-100 in PBS, and incubated in blocking solution containing primary antibodies overnight at 4ºC. Sections were then washed with PBS and incubated with secondary antibodies diluted in blocking solution for 1 hour at room temperature. Ductal epithelium was detected using rhodamine-labeled Dolichos Biflorus Agglutinin (DBA) (1:200, Vector Laboratories RL-1032) applied during secondary antibody incubation. Sections were washed in PBS and mounted in Fluoromount Aqueous Mounting Medium (Sigma Aldrich, F4680) containing 100µg/ml DAPI. Images representing 1µm optical slices were taken using a Zeiss LSM 700 confocal microscope equipped with 405, 488, 555, and 633 nm lasers.

For morphometric analyses of islets, 10µm thick sections of pancreata were collected every 200µm covering the entire pancreas, immunostained for insulin and glucagon, and imaged on a Zeiss AxioImager. Islet areas were determined using the ImageJ software (NIH).

Islet isolation, and RT-PCR analysis

Islets were isolated as previously described (Wollheim et al., 1990). Briefly, pancreata collected from 1-2 month old mice were distended using collagenase (Collagenase P, 0.375 mg/ml, Roche) dissolved in Hank's Balanced Salt Solution (HBSS, Mediatech), and digested at 37°C. Digested pancreata were washed with HBSS, and subjected to discontinuous density gradient centrifugation using varying histopaque densities (Sigma-Aldrich). The islet layer was collected, washed with HBSS, and islets handpicked under an inverted microscope.

Islets from 1-2 month old mice or the total pancreas from embryonic day 15 mice were collected, lysed in Trizol reagent (Invitrogen), and RNA purified using RNeasy micro columns (Qiagen). RNA was then reverse transcribed using a RETROscript kit (Ambion), and RT PCR was carried out using the primers TrkA-Fwd: 5'-TCCAAGTCAGCGTCTCCTTCCCA, TrkA-Rev: 5'-AGCCGTTGAACAACCAGCGCA, NGF-Fwd: 5'-GGGAGCGCATCGAGTTTTG, NGF-Rev: 5'-CACCTCCTTGCCCTTGATGT, S18-Fwd: 5'-TTCCGCAAGTTCACCTACC, S18-Rev: 5'-CGGGCCGGCCATGCTTTACG.

CHAPTER 2. DIRECT AND INDIRECT ROLES FOR NGF IN PANCREATIC ISLETS

Portions of this chapter were previously submitted for publication at Developmental Cell: Jessica Houtz, Philip Borden, Alexis Ceasrine, and Rejji Kuruvilla (2016). *Neurotrophin signaling is required for glucose-induced insulin secretion.*

INTRODUCTION

NGF and TrkA in the pancreas

NGF has previously been proposed to directly influence β-cell development and function. Early studies found that application of NGF to fetal β-cells or β-cell lines led to the extension of neurite-like processes, increased biosynthesis of insulin, and increased production of the neurotransmitter, GABA (Polak et al., 1993, Vidaltamayo et al., 1996, Gonzalez del Pliego et al., 2001, Navarro-Tableros et al., 2004). Work performed in single dissociated rat β-cells has further demonstrated that NGF is capable of eliciting an increase in glucose stimulated insulin secretion and Ca2+ currents (Rosenbaum et al., 2001, Rosenbaum et al., 2002, Frodin et al., 1995).

Similar to its key role in neuronal development, NGF has also been implicated in β-cell survival. NGF depletion using a neutralizing antibody was shown to result in inhibition of PI3K signaling and activation of c-Jun kinase and subsequent apoptosis in both human islets and a β-cell line (Pierucci et al., 2001). In another investigation, induction of β-cell death with streptozotocin was somewhat diminished by the application of 4-methylcatechol which stimulates NGF production (Gezginci-Oktayoglu and Bolkent, 2011).

Although some studies indicate that the source of NGF in the pancreas may be supplied by non-endocrine cells surrounding β-cells (Miralles et al., 1998, Kanaka-Gantenbein et al., 1995a), other bodies of work have demonstrated that NGF could be produced by β-cells themselves *in vitro* (Hiriart et al., 2001, Rosenbaum et al., 1998), implying an autocrine signaling mechanism. It is possible that, under culture conditions,

dissociated β-cells are stimulated to produce limited amounts of NGF, but there has been very little analysis of regulated NGF secretion *in vivo*. Interestingly, a study comparing the regulation of TrkA and p75 in islets versus a β-cell line found that glucose elicits opposite effects on neurotrophin receptor expression in the different systems. In the Ins1 β-cell line, TrkA expression decreases with increasing concentrations of glucose in culture, whereas, glucose has a stimulatory effect on islet expression of neurotrophin receptors and Ins1 expression of p75 (Raile et al., 2006). In this study we define a previously unrecognized role for paracrine NGF signaling from intra-islet pericytes and vascular smooth muscle cells in large diameter blood vessels in regulating insulin secretion from islets.

Neuron-target interactions during development

The effects of neurotrophin signaling have been best studied in the developing nervous system where they have been shown to influence neuron proliferation and survival, axon growth and branching, and synaptic plasticity (Huang and Reichardt, 2001a, Chao et al., 2006). The timing of neurotrophin dependent development of neurons often coincides with critical developmental time points in non-neuronal systems, and there is mounting evidence that neurons participate in the development of the tissues they innervate. A classic example of this neuron-target interaction is the development of the neuromuscular junction (Sanes and Lichtman, 1999). More recently, studies have established additional roles for peripheral innervation in mediating the development of a variety of targets including the salivary glands (Knox et al., 2010, Nedvetsky et al., 2014), bone (Fukuda et al., 2013), and immune system (Tian et al., 2011). In a recently published study, we identified sympathetic signaling through beta-adrenergic receptors as a key regulator of pancreatic islet

architecture during development and β-cell function (Borden et al., 2013). We employed a genetic approach to delete TrkA specifically in neurons, accomplished by crossing mice expressing Cre recombinase under the control of the tyrosine hydroxylase promotor (TH-Cre) with conditional, floxed TrkA mice. The resulting *TH-Cre;TrkA$^{f/f}$* mice have no or vastly attenuated sympathetic innervation of peripheral tissues, including the pancreas. We found that these mice have pancreatic islets with severe disruptions in islet architecture and impaired glucose tolerance and insulin secretion. However, since *TH-Cre;TrkA$^{f/f}$* mice also exhibit a loss of sensory neurons that briefly express TH during development and rely on TrkA signaling for survival, my contribution to this study (detailed in the Results section below) was to use a chemical sympathectomy approach to elucidate the role of sympathetic innervation in islet organization.

RESULTS

Neurotrophin signaling directly affects adult islet function

Given our expression analysis in *NGF$^{LacZ/+}$* mice (see Chapter 1, Fig. 1-2) we hypothesized that NGF coming from smooth muscle contractile cells, and not β-cells themselves, might be contributing to neurotrophin signaling in the adult. To this end we employed a genetic mouse model in which we can spatially and temporally ablate NGF only in large diameter blood vessels and pericytes in the adult.

Conditional ablation of NGF in adult vasculature impairs glucose homeostasis and insulin secretion

Previous studies have indicated that NGF positively influences adult β-cell function and survival (Rosenbaum et al., 2002, Pierucci et al., 2001, Hata et al., 2015). However, these results were obtained using *in vitro* methods in which exogenous NGF is added to cultured β-cells. Utilizing mouse genetics, we provide the first *in vivo* analysis of NGF function in the adult pancreas. In order to ablate NGF in adult smooth muscle cells, we crossed mice expressing a Myh11-Cre^{ER} transgene, which has been shown to be specifically expressed in smooth muscle contractile cells and pericytes (Wirth et al., 2008, Joyce et al., 1985), with conditional *NGF$^{f/f}$* mice (Muller et al., 2012). We confirmed expression of Myh11-Cre^{ER} in the relevant vascular cells in the pancreas by co-localization of Myh11-Cre^{ER}; reporter expression and immunostaining for β-galactosidase in *NGF$^{LacZ/+}$;Myh11-Cre^{ER} (R26-YFP)* double mutant mice two weeks after injection with tamoxifen **(Fig. 2-4A,A')**. Furthermore, qPCR analysis of NGF expression in isolated islets

from *Myh11-CreER;NGF$^{f/f}$* conditional mice revealed a 64% reduction in tamoxifen treated mice compared with vehicle treated mice **(Fig. 2-4B)**. When we evaluated metabolic parameters in conditional NGF mutant mice, we found that tamoxifen treated *Myh11-CreER;NGF$^{f/f}$* have impaired glucose tolerance and reduced insulin secretion *in vivo* **(Fig. 2-4C,D)**. In addition to whole-animal defects in glucose tolerance and insulin secretion, we found a similar impairment in GSIS *in vitro* in islets isolated from vehicle and tamoxifen treated *Myh11-CreER;NGF$^{f/f}$* mice **(Fig. 2-4E)**.

Pancreatic NGF does not contribute to β-cell function

While our findings in tamoxifen treated *Myh11-CreER;NGF$^{f/f}$* conditional mice strongly support a model in which the local source of NGF in the pancreas that is important for β-cell function comes from vascular smooth muscle, others have suggested that β-cells synthesize and secrete NGF to promote autocrine neurotrophin signaling within islets (Rosenbaum et al., 1998, Vidaltamayo et al., 2003). Given these previous findings, and our own *in vitro* data demonstrating expression of β-galactosidase *in vitro* in β-cells from *NGF$^{LacZ/+}$* reporter mice, we tested the function of NGF in the pancreas. *Pdx1-Cre;NGF$^{f/f}$* were generated by crossing NGF conditional mice with mice expressing Cre recombinase under the control of Pdx1, a transcription factor expressed in pancreatic progenitor cells (Hingorani et al., 2003). *Pdx1-Cre;NGF$^{f/f}$* mice would be expected to have an early and pancreas-wide deletion of NGF. However, when we performed qPCR analysis on islets isolated from *Pdx1-Cre;NGF$^{f/f}$* and *NGF$^{f/f}$* mice, we found no significant reduction in NGF expression **(Fig. 2-5A)**. This result is in contrast with the reduction in islet NGF expression we found in *Myh11-CreER;NGF$^{f/f}$* mice, and strongly suggests that vascular smooth muscle

is the relevant source of NGF in the pancreas. In further support of this notion, glucose tolerance is no different between *Pdx1-Cre;NGF$^{f/f}$* and *NGF$^{f/f}$* mice **(Fig. 2-5B)**, confirming that autocrine NGF signaling does not play an important role *in vivo* in maintaining glucose homeostasis.

Neurotrophin signaling indirectly influences islet biology through its effects on sympathetic innervation

While the focus of this thesis is on a direct role for neurotrophin signaling in the pancreas in β-cell function, I have also contributed to work that defines a role for sympathetic innervation in the proper development of islet architecture and function. Since NGF is a vial to the survival of sympathetic neurons that innervate islets, neurotrophin signaling can be viewed as having an indirect impact on β-cell development and function via its influence on pancreatic innervation. In addition to sympathetic neurons, there are a population of sensory neurons that transiently express TH early during development and also rely of TrkA for survival. We found a loss of both sensory and sympathetic innervation in islets from *TH-Cre;TrkA$^{f/f}$* mice that was accompanied by alterations in islet architecture and impaired glucose tolerance in adulthood (Borden et al., 2013). In order to dissect which peripheral neurons were responsible for instructing islet formation I performed chemical sympathectomy using the drug 6-hydroxydopamine (6-OHDA). 6-OHDA is an isomer of norepinephrine that functions as a neurotoxin in sympathetic neurons where it is selectively taken up by the vesicular monamine transporter 2 (VMAT2). Once inside neurons, 6-OHDA is oxidized to produce free radicals that destroy nerve terminals.

Starting from birth (P0), we treated postnatal mouse pups with 6-OHDA for 6 days to destroy sympathetic innervation. I observed a selective loss of TH fibers innervating islets in postnatal day 6 (P6) mice **(Fig. 2-3A,B)**, while CGRP expressing sensory fibers remained intact **(Fig. 2-3C,D)**. Similar to the defects seen in islet architecture in *TH-Cre;TrkA$^{f/f}$* animals, I found irregular pancreatic islet shapes **(Fig. 2-4A,B,E)** and mislocalization of alpha cells in animals treated with 6-OHDA **(Fig. 2-4C,D,F)**. When allowed to age to one month, animals injected with 6-OHDA showed normal glucose tolerance **(Fig. 2-5)**. These data are in contrast with the obvious defects in glucose tolerance observed in *TH-Cre;TrkA$^{f/f}$* mice, and additional analyses will be necessary to determine if sympathetic innervation remains absent by one month of age, after the last treatment of 6-OHDA at P6. Reports of axon regeneration following 6-OHDA treatment suggest that the normal glucose tolerance I observed could be due to restoration of sympathetic innervation by this time. Alternative possibilities are that sensory innervation contributes to normal β-cell function, or that sympathetic innervation is important during an early developmental window that precedes the postnatal time period.

Figure 2-1: Conditional deletion of NGF in the adult vasculature results in impaired glucose tolerance and insulin secretion.

(A,A') Expression of *Myh11-Cre^{ER} (R26-YFP)* in *NGF$^{LacZ/+}$* (β-gal immunostaining) smooth muscle cells and intra islet pericytes scale bars, **(A)** 100µm, **(A')** 50µm. **(B)** qPCR analysis reveals a 64% deletion of NGF in islets from *Myh11-CreER;NGF$^{f/f}$* mice treated with tamoxifen (Tmxfn) compared to vehicle. Values are the mean ± SEM from n=4 independent experiments. ∗∗p<0.01, t-test. **(C)** Loss of NGF in adult vasculature results in impaired glucose tolerance in *Myh11-CreER;NGF$^{f/f}$* mice treated with tamoxifen. Values are mean ± SEM for n=7 vehicle and n=7 tmxfn injected mice. *p<0.05. **(D)** *In vivo* glucose-stimulated insulin secretion is attenuated by loss of NGF in *Myh11-CreER;NGF$^{f/f}$* mice. Values are mean ± SEM for n=12 vehicle and n=12 tmxfn injected mice.*p<0.05, **p<0.01, *t*-test. **(E)** Glucose stimulated insulin secretion is attenuated in *Myh11-CreER;NGF$^{f/f}$* mice treated with tamoxifen compared to vehicle. Values are means ± SEM from n=8 Vehicle and n=8 Tmxfn treated animals. *p<0.05, **p<0.01, two-way ANOVA with Bonferroni post-hoc test

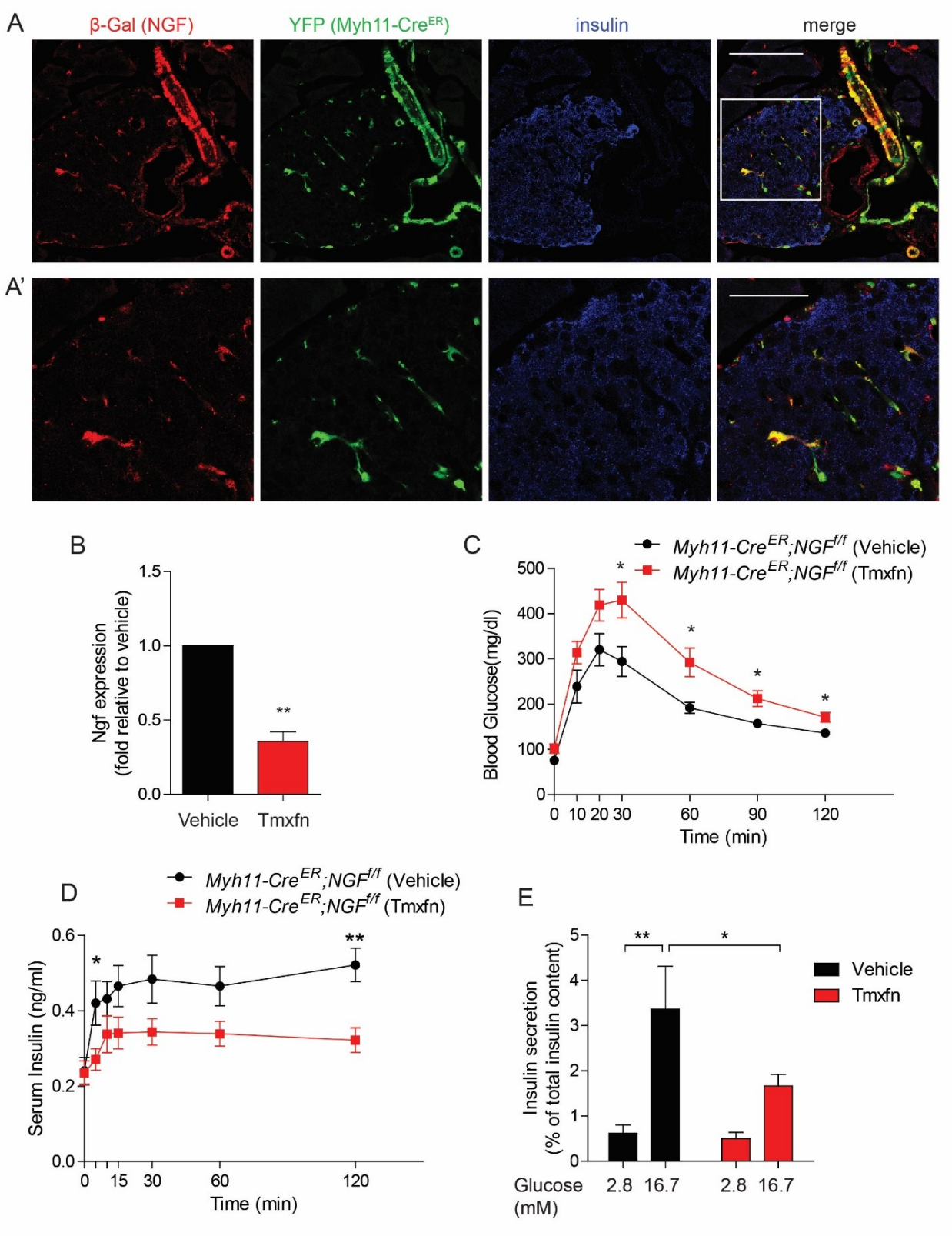

A
β-Gal (NGF)
YFP (Myh11-CreER)
insulin
merge
A'
B
Ngf expression (fold relative to vehicle)
1.5
1.0
0.5
0.0
**
Vehicle
Tmxfn
C
Myh11-CreER;NGFf/f (Vehicle)
Myh11-CreER;NGFf/f (Tmxfn)
Blood Glucose(mg/dl)
500
400
300
200
100
0
*
0 10 20 30 60 90 120
Time (min)
D
Myh11-CreER;NGFf/f (Vehicle)
Myh11-CreER;NGFf/f (Tmxfn)
Serum Insulin (ng/ml)
0.6
0.4
0.2
0.0
* **
0 15 30 60 120
Time (min)
E
Insulin secretion (% of total insulin content)
5
4
3
2
1
0
** *
Vehicle
Tmxfn
Glucose (mM)
2.8 16.7 2.8 16.7

Figure 2-2: Conditional deletion of NGF in the pancreas does not influence glucose homeostasis

(A) Expression of NGF in islets from *Pdx1-Cre;NGF$^{f/f}$* mice is not reduced compared to *NGF$^{f/f}$* littermates by qPCR analysis. Values are means ± SEM for n =2 NGFf/f and n=2 *Pdx1-Cre;NGF$^{f/f}$* difference is not significant by one-sample t-test. **(B)** Glucose tolerance is normal in *Pdx1-Cre;NGF$^{f/f}$*. Values are means ± SEM from n=10 *NGF$^{f/f}$* and n=10 *Pdx1-Cre;NGF$^{f/f}$* animals.

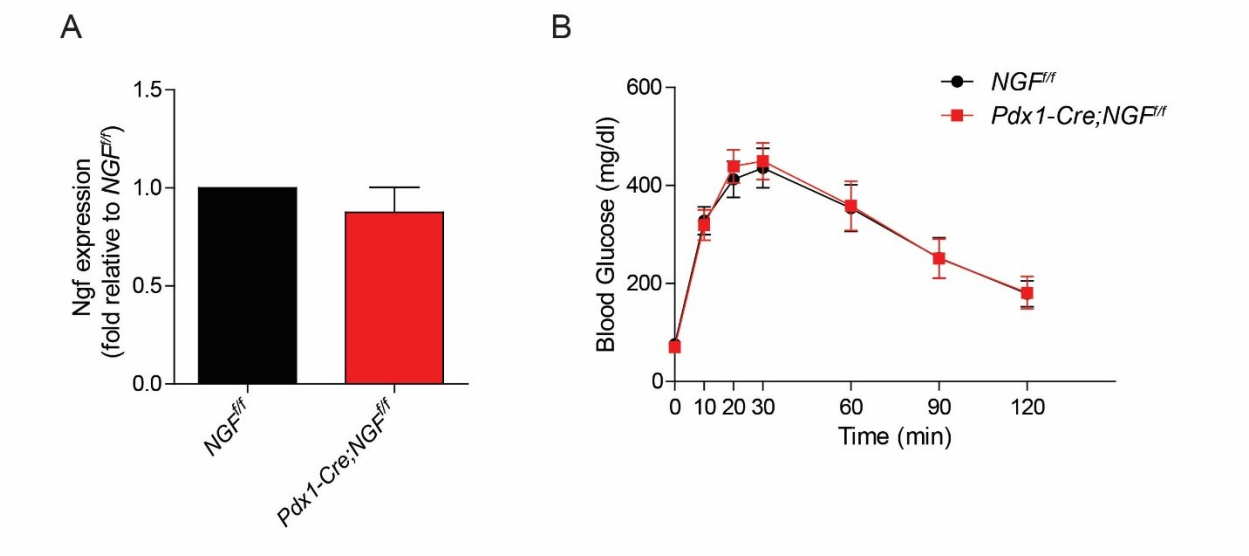


Figure 2-3: 6-OHDA selectively abolishes sympathetic innervation but not sensory innervation of islets in postnatal day 6 mice.

(A and B) Treatment of newborn mice with 6-OHDA daily until postnatal day 6 (P6) results in depletion of sympathetic fibers (arrows) in the pancreas. **(C,D)** Mice treated with 6-OHDA have normal sensory innervation of islets. Scale bars, 50 μm.

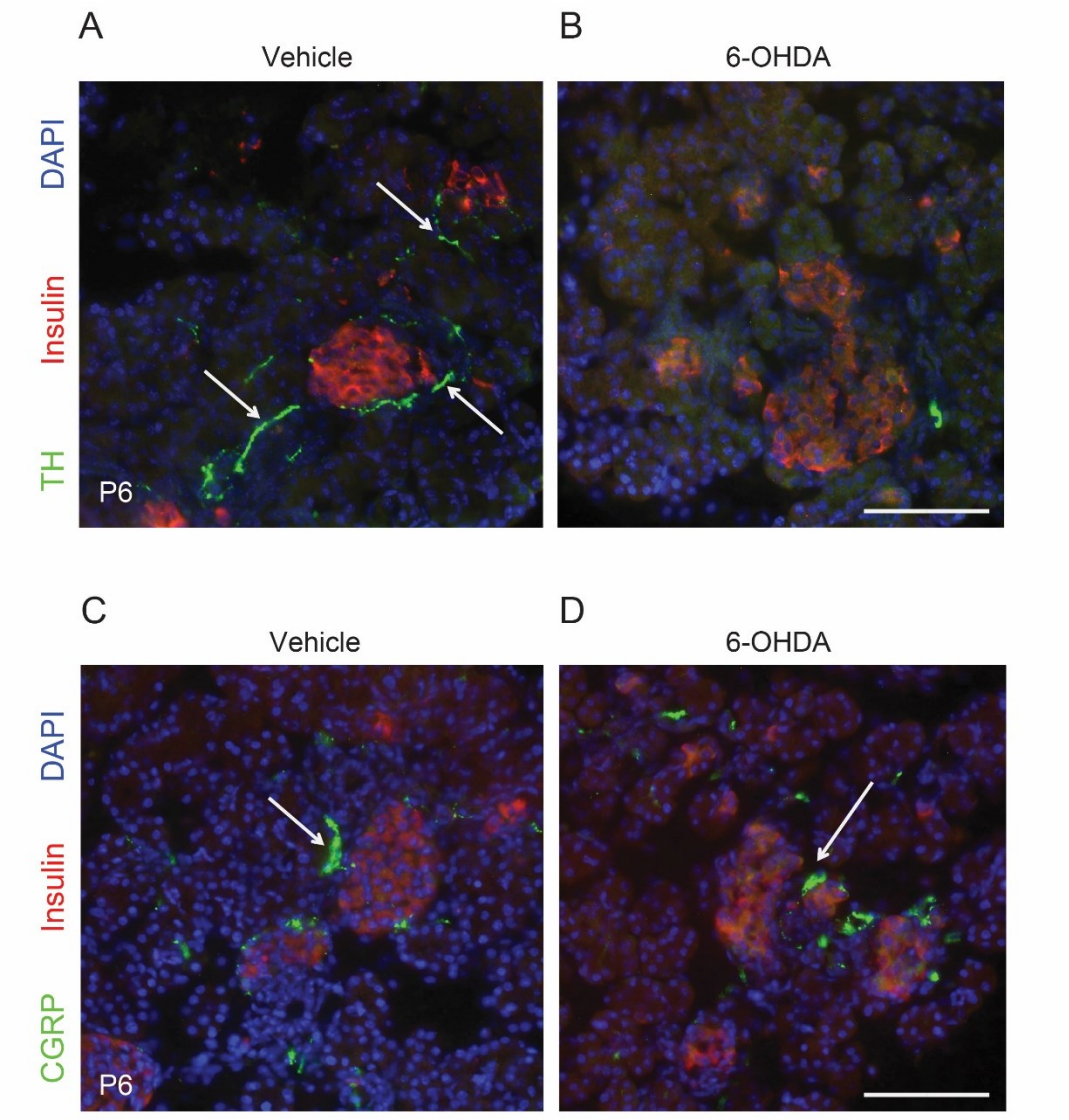


Figure 2-4: Selective chemical sypathectomy with 6-OHDA results in impaired islet architecture

(A, B) Chemical sympathectomy with 6-OHDA administration disrupts islet clustering in neonatal MIP-GFP mice. GFP+ β cells (green) and DAPI (nuclei) staining are shown. Scale bars, 100μm. **(C,D)** Immunostaining for islet hormones shows disrupted arrangement of endocrine cells within islets in neonatal (P6) mice treated with 6-OHDA. **(E)** Islet shape is quantified by circularity index, and **(F)** percentage of islets with mis-localized α cells in P6 mice treated with 6-OHDA compared with vehicle. Values are mean ± SEM from 5 control and 3 mutant animals, *$p<0.05$, t-test. Philip Borden performed quantifications represented in **(E)** and **(F)**.

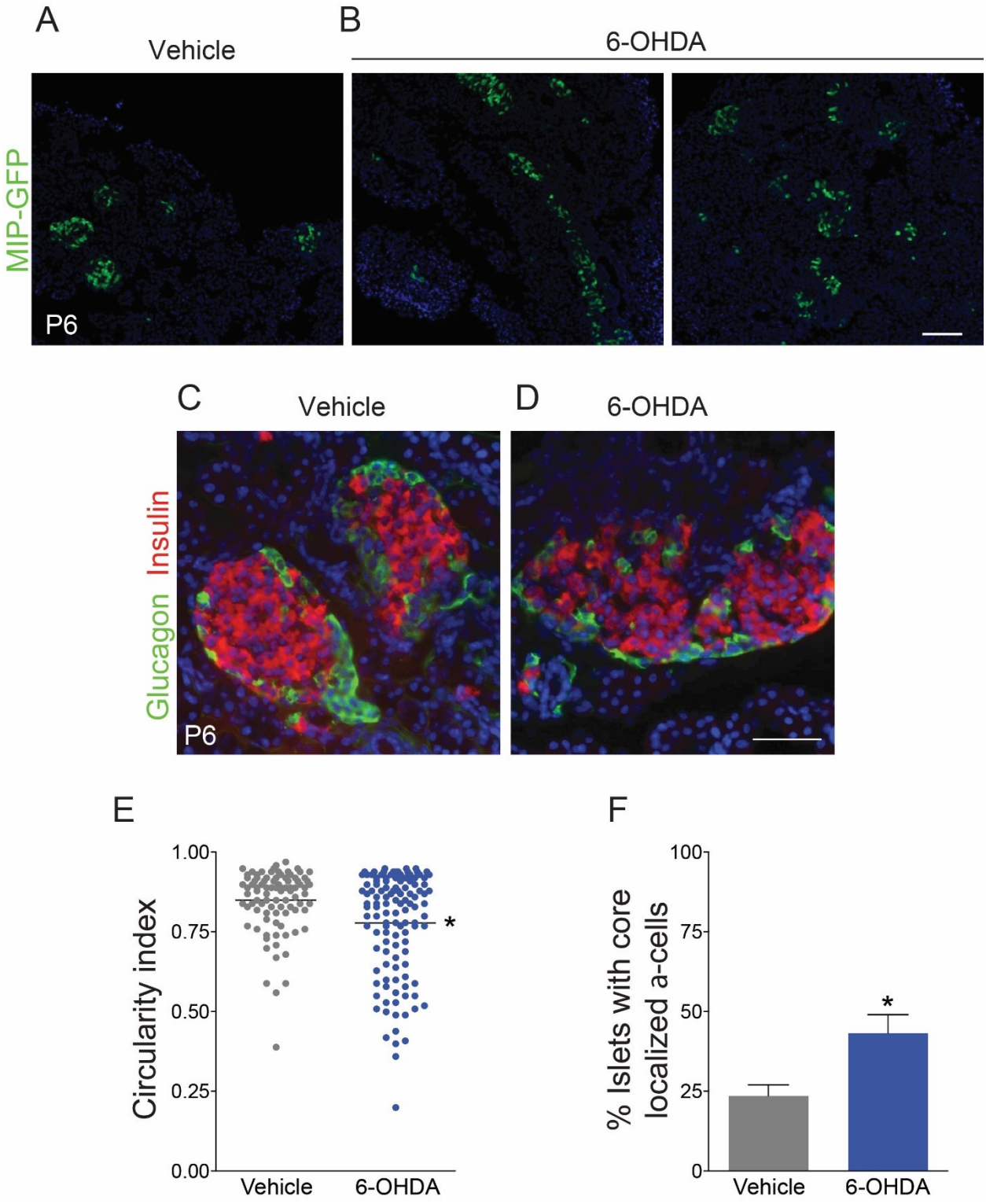
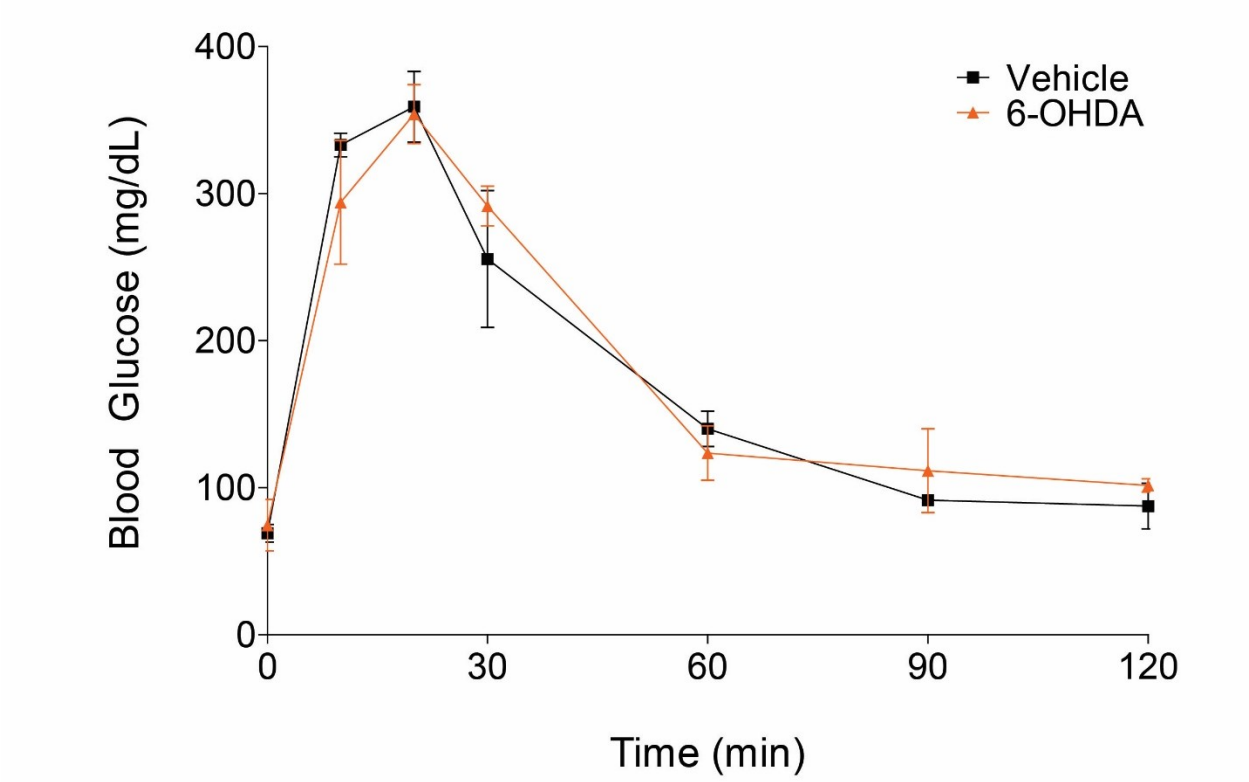

A
Vehicle
B
6-OHDA
MIP-GFP
P6
C
Vehicle
D
6-OHDA
Glucagon
Insulin
P6
E
Circularity index
1.00
0.75
0.50
0.25
0.00
*
Vehicle
6-OHDA
F
% Islets with core localized a-cells
100
75
50
25
0
*
Vehicle
6-OHDA

Figure 2-5: Postnatal administration of 6-OHDA does not appear to impair glucose tolerance in adulthood

Mice were injected with glucose (2g/kg body weight) delivered intra-peritoneally (i.p.), after an overnight (16 hour) fast. Values are the mean ± SEM for n=2 vehicle and n=2 6-OHDA injected mice.



DISCUSSION

Our work defines a direct role for NGF in mediating glucose homeostasis and insulin secretion. Excitingly, we provide the first *in vivo* evidence that NGF is important for maintaining glucose homeostasis in the adult **(Fig. 2-1)**. The defects in glucose homeostasis and insulin secretion we see in *Myh11-CreER;NGF$^{f/f}$* mice, together with the NGF expression pattern **(Chapter 1 Fig. 1-2 and Fig. 2-1)**, imply that vascular signals derived from large diameter blood vessels juxtaposed to islets should be sufficient to support adult islet function. Consistent with this notion, recent studies have shown that regression of intra-islet microvasculature due to loss of VEGF signaling did not perturb adult insulin secretion, and elicited only modest elevations in blood glucose (Reinert et al., 2013, D'Hoker et al., 2013). While the precise role of pericytes in islet function is unclear, these contractile cells are known to be important for maintaining vascular stability throughout the body (Hellstrom et al., 2001), and a dramatic hyperplasia of intra-islet pericytes accompanied by thickening of the capillary basement membrane and peri-capillary fibrosis in the pancreas is observed in models of both type 1 (Tang et al., 2013) and type 2 diabetes (Hayden et al., 2008, Dai et al., 2013, Nakamura et al., 1995). Our results suggest that pericytes provide crucial paracrine signaling to maintain islet function, beyond their established role in maintaining microvascular stability.

Neurotrophin signaling has been best studied in the classical sense where targets provide trophic support to innervating neurons. Interestingly, there is growing evidence that reciprocal interactions occur in which neurons shape the development of their target tissues (Fukuda et al., 2013, Knox et al., 2010, Tian et al., 2011). We demonstrate a

previously unappreciated, indirect role for neurotrophin signaling in shaping islet architecture via sympathetic innervation. Since chemical ablation of the sympathetic nervous system starting at birth effectively altered islet architecture, but did not impair glucose tolerance in mice, it is likely that neuron-target interactions influence islet development at very early stages in development. These findings also raise the intriguing possibility that functional deficits seen in genetic sympathectomized mice are not necessarily a consequence of disrupted islet architecture. Future studies also investigating critical windows of development would be of use in determining when sympathetic innervation is important for different aspects of islet biology.

Given the significance of NGF in determining the extent of islet innervation, and the importance of sympathetic innervation in shaping islet development and function, it would be very interesting to look at the patterns of innervation in *Pdx1-Cre;NGF$^{f/f}$* mice and tamoxifen-treated *Myh11-CreER;NGF$^{f/f}$* mice at different stages of development. Although our data suggests that pancreas-derived NGF doesn't play a critical role in islet function, pancreas-wide loss of NGF would be expected to reduce sympathetic innervation to acinar and ductal cell types as well, which may influence the exocrine function of the pancreas. Furthermore, there are reported differences in sympathetic innervation of human versus mouse islets (Dolensek et al., 2015, Rodriguez-Diaz et al., 2011), which may reflect species-specific differences in patterns of NGF expression in the pancreas. While sympathetic nerves in mouse islets have been shown to primarily innervate the mantle of islets, with most contacts being made on glucagon secreting alpha cells, there is only sparse innervation of endocrine cells in human islets with most contacts being made on vascular smooth muscle cells (Dolensek et al., 2015). It is curious that although vascular cells appear

to provide the greatest source of NGF in the pancreas, sympathetic neurons in mice do not innervate intra-islet vascular cells to the extent seen in human islets. Future studies will be important in determining how pancreas-specific and vascular-specific deletion of NGF influences innervation and morphology of the pancreas during development. It is possible that smooth muscle cells undergo important changes in the regulation of NGF secretion during development that confer differences in innervation versus paracrine signaling to β-cells in the adult.

METHODS

Mice

All procedures relating to animal care and treatment conformed to Johns Hopkins University Animal Care and Use Committee (ACUC) and NIH guidelines. Mice were housed in a standard 12:12 light-dark cycle. Mice were maintained on a *C57BL/6* background, or mixed *C57BL/6* and *129P*, or *C57BL/6* and *FVB* backgrounds. Both sexes were used for analyses at 1-2 months of age, unless stated otherwise in the figure legends. MIP-GFP (strain no: 006864) and Myh11-CreER (strain no: 019079) were obtained from Jackson Laboratory, and *Pdx1-Cre* mice (*Tg(Pdx1-Cre^{Tuv})* strain no: 01XL5) mice were obtained from NCI Frederick mouse repository. Floxed NGF (*NGF$^{f/f}$*) mice were a generous gift from Dr. Liliana Minichiello (Oxford) and *NGF$^{LacZ/+}$* mice were a generous gift from Dr. David Ginty (Harvard).

6-OHDA Injections

Beginning on the day of birth (P0), *TrkAf/f* mice were injected i.p. once daily with 250 mg/kg of 6-OHDA (Sigma-Aldrich) freshly dissolved in 0.1% ascorbic acid (Sigma-Aldrich) in 0.9% saline (Sigma-Aldrich) or with ascorbic acid-saline solution as a control. Pups were sacrificed at P6 and pancreata processed for TH, CGRP, insulin, and glucagon immunofluorescence. For glucose tolerance testing, mice were aged to 1 month after the last 6-OHDA injection.

Tamoxifen Injections:

Beginning at 5-6 weeks of age, *Myh11-Cre^{ER}(R26-YFP);NGF$^{LacZ/+}$* or *Myh11-Cre^{ER};NGF$^{f/f}$* or mice were injected subcutaneously with 180mg/kg tamoxifen (Sigma) in corn oil, or corn oil alone, for 5 days. 2-3 weeks after the last injection, vehicle and tamoxifen mice underwent glucose tolerance and insulin secretion testing.

Antibodies

Primary antibodies for immunofluorescence include guinea pig anti-insulin (1:300, abcam ab7842), mouse anti-glucagon (1:400, abcam ab10988), rabbit anti-TH (1:500, MilliporeAB152), rabbit anti-CGRP (1:500, Sigma-Aldrich C8198), and chicken anti-beta-galactosidase (1:100, Millipore AB3403-I). Fluorescent secondary antibodies were Alexafluor-350, -488,-546, or -647-conjugated and used at 1:200 (Invitrogen)

Immunofluorescence

Tissues were fixed overnight in 4% paraformaldehyde (PFA) (Sigma) at 4ºC, cryoprotected in 30% sucrose overnight, frozen in OCT, and 10 µm thick sections were collected. Sections were permeabilized in PBS with 1% Triton X-100, blocked in 5% goat serum with 0.1% Triton X-100 in PBS, and incubated in blocking solution containing primary antibodies overnight at 4ºC. Sections were then washed with PBS and incubated with secondary antibodies diluted in blocking solution for 1 hour at room temperature. Sections were washed in PBS and mounted in Fluoromount Aqueous Mounting Medium (Sigma Aldrich, F4680) containing 100µg/ml DAPI. Images representing 1µm optical slices were taken using a Zeiss LSM 700 confocal microscope equipped with 405, 488, 555, and 633 nm lasers.

Morphometric analyses of islets

10μm thick sections of pancreata were collected every 200μm and immunofluorescently labeled for insulin and glucagon. Islet circularity was determined using the ImageJ software (NIH) shape descriptors tool, calculated by the equation: circularityindex=4π(area/perimeter2) with '1' and '0' denoting a perfect circle and an increasingly elongated polygon, respectively. For cytoarchitecture analyses, an islet was considered to have increased α-cell core localization if more than three α-cells were localized to greater than two cell layers inside of the outer islet perimeter.

Islet isolation, and quantitative RT-PCR analysis

Islets were isolated as previously described (Wollheim et al., 1990). Briefly, pancreata collected from adult mice were distended using collagenase (Collagenase P, 0.375 mg/ml, Roche) dissolved in Hank's Balanced Salt Solution (HBSS, Mediatech), and digested at 37°C. Digested pancreata were washed with HBSS, and subjected to discontinuous density gradient centrifugation using varying histopaque densities (Sigma-Aldrich). The islet layer was collected, washed with HBSS, and islets handpicked under an inverted microscope.

Islets were collected, lysed in Trizol reagent (Invitrogen), and RNA purified using RNeasy micro columns (Qiagen). RNA was then reverse transcribed using a RETROscript kit (Ambion). Quantitative RT PCR was carried out using SYBR geen mix (BioRad) in an Applied Biosystems StepOnePlus RT PCR cycler instrument, using the primers: NGF-Fwd: 5'-GACTCCAAACACTGGAACTCATACTG, NGF-Rev: 5'-

GCCTGCTTCTCATCTGTTGTCA, 18s-Fwd: 5'- CGCCGCTAGAGGTGAAATTC, 18s-Rev: 5'- TTGGCAAATGCTTTCGCTC. Fold change in *NGF* transcript levels was calculated using the -2ΔΔCt method, normalizing to *18S rRNA* transcript.

In vivo analyses of glucose tolerance, and glucose induced insulin secretion

For glucose tolerance tests, 1-2 month-old mice were fasted overnight, with a blood glucose reading the evening before the assay serving as a fed blood glucose measurement. The next morning, mice were injected with glucose (2g/kg, i.p). Blood glucose measurements were made from tail blood using a OneTouch Ultra glucometer at the indicated times (Gu et al., 2010). For acute treatments with 1NMPP1, mice received i.p. injections with 20ng/g 1NMPP1 or DMSO, 20 minutes prior to glucose administration.

For *in vivo* insulin secretion assays, mice were fasted overnight before being injected with glucose (3 g/kg, i.p.). Blood was collected from the tail at the times indicated, spun down, and the resulting plasma fractions subjected to insulin ELISA (Crystal Chem, 90080). Reactions were assessed using a Tecan infinite 200 plate reader.

In vitro GSIS assays

For insulin secretion assays in isolated islets, harvested islets were allowed to recover overnight in RPMI 1640 media containing 5% fetal bovine serum (FBS) and 5 U/l penicillin/streptomycin. Islets were washed in Krebs-Ringer HEPES buffer (KRHB) containing low (2.8 mM) glucose and allowed to stabilize for 1 hour. Groups of 5-10 islets were then handpicked into 24-well dishes and further incubated in low (2.8 mM) or high

glucose (16.7 mM) in KRHB buffer for another 30 minutes. Supernatant fractions were removed, the islets were lysed in acid ethanol, followed by insulin ELISA (Crystal Chem) to determine the insulin concentrations in both supernatant and islet fractions.

CHAPTER 3. TRKA SIGNALING IN β-CELLS IS NECESSARY FOR GLUCOSE HOMEOSTASIS AND INSULIN SECRETION

Portions of this chapter were previously submitted for publication at Developmental Cell: Jessica Houtz, Philip Borden, Alexis Ceasrine, and Rejji Kuruvilla (2016). *Neurotrophin signaling is required for glucose-induced insulin secretion.*

INTRODUCTION

Neurotrophin signaling in β-cells

A potential intrinsic role for neurotrophins in the development and function of the pancreas has been realized for almost 25 years, although *in vivo* evidence and detailed mechanisms have been lacking. Reports of TrkA expression have been made for a variety of cell types in both the exocrine and endocrine pancreas during early and adult stages of development (Shibayama and Koizumi, 1996, Sariola, 2001, Miralles et al., 1998, Kanaka-Gantenbein et al., 1995b, Scharfmann et al., 1993). All studies, including our results, agree that β-cells express TrkA, and subsequent work has focused on the effect of NGF on β-cell development and insulin secretion.

Work performed in single dissociated rat β-cells has further demonstrated that NGF is capable of eliciting an increase in glucose stimulated insulin secretion and Ca2+ currents (Rosenbaum et al., 2001, Rosenbaum et al., 2002, Frodin et al., 1995). Long-term exposure to NGF in these experiments has been proposed to increase calcium currents via an increase in the synthesis of calcium channels, while a short 5 minute pulse of NGF can increase calcium conductance by stimulating the movement of channels to the cell surface, or modulating their activity through phosphorylation (Rosenbaum et al., 2002). In neonatal rat β-cells, increased calcium conductance in response to NGF can be attributed to both an increase in the surface localization of the α1c VGCC subunit and to an increase in the expression of the β2 VGCC that is enriched in adult β-cells (Navarro-Tableros et al., 2007). Besides calcium channels, sodium channels are also thought to contribute to the electrical excitability of β-cells. In rat β-cells, NGF increases the expression of type III sodium

channel α subunits, and enhances insulin secretion which is sensitive to the sodium channel blocker, tetrodotoxin. These effects are likely mediated by signaling through TrkA, as application of the selective tyrosine kinase inhibitor, K252a, was shown to block NGF-induced increases in insulin secretion in dissociated rat β-cells (Rosenbaum et al., 2001).

While these studies convincingly demonstrate that NGF is capable of eliciting changes in the electrical properties of single dissociated rat β-cells, there are several caveats to the stated conclusions. First, the kinetics of insulin secretion between rat and mouse islets is known to differ (Ma et al., 1995), and sodium channels are thought to have a greater contribution to electrical activity in rat β-cells than either mouse or human β-cells (Misler et al., 1992). Second, it is not clear what significance these findings have in the context of insulin secretion in intact islets or *in vivo*. Here, we provide the first *in vivo* evidence that TrkA signaling in β-cells is required for normal glucose stimulated insulin secretion (GSIS). Furthermore, we demonstrate that acute TrkA activity in the adult mouse is necessary for β-cell function and glucose homeostasis.

RESULTS

TrkA-deficient mice exhibit impaired glucose tolerance and reduced insulin secretion

It is clear that neurotrophin signaling has an indirect role in β-cell development via its role in promoting the proper development of the peripheral nervous system, however, it is less evident how neurotrophin signaling could directly impact β-cell development and function. To address the intrinsic role of TrkA in endocrine cells, we mated TrkAf/f with mice expressing Cre recombinase under the control of Pdx1, a transcription factor expressed in pancreatic progenitor cells. *Pdx1-Cre;TrkA$^{f/f}$* mice would be expected to have an early and pancreas-wide deletion of TrkA. Biotinylated NGF ligand binding was markedly reduced in sections of pancreas from mutant islets **(Fig. 3-1A,B),** indicative of efficient TrkA deletion. In data previously published a thesis by Philip Borden, *Pdx1-Cre;TrkA$^{f/f}$* mice have been shown to survive to adulthood, have no gross morphological abnormalities, and have normal body weight (Borden, 2013). When we assessed metabolic parameters at the whole animal level, 1.5-2 month-old *Pdx1-Cre;TrkA$^{f/f}$* mice were slightly hyperglycemic when fed ad libitum although fasted blood glucose levels were normal **(Fig. 3-1C,D)**. However, the *Pdx1-Cre;TrkA$^{f/f}$* mice showed significant glucose intolerance with glucose administration (intra-peritoneally), and decreased circulating insulin levels compared to control *TrkA$^{f/f}$* mice **(Fig. 3-1E,F)**. Previous analysis of islet formation and cyto-architecture in neonatal *Pdx1-Cre;TrkA$^{f/f}$* mice revealed no abnormalities however, mutant islets showed a modest decrease in size that was only apparent at one month of age

and this may be due, in part, to the trophic effects of NGF signaling on β-cell survival (Borden, 2013).

Although restricted Cre recombinase activity has been reported in extra-pancreatic tissues, including the hypothalamus, in Pdx1-Cre transgenic mice (Song et al., 2010), there is little overlap with TrkA expression (Fagan et al., 1997). Additionally, the Pdx1-CreTuv transgenic mice that we employed do not carry a human growth hormone mini-gene, commonly found in several Cre lines, that elicits metabolic defects. Together, these results suggest that loss of TrkA in pancreatic tissues causes the defects in glucose homeostasis and insulin secretion in *Pdx1-Cre;TrkA$^{f/f}$* mice.

TrkA kinase activity is acutely required for GSIS

In *Pdx1-Cre;TrkA$^{f/f}$* mice, deletion of TrkA is initiated at early embryonic stages. Thus, the observations of impaired glucose tolerance and reduced insulin secretion in these mice could stem from developmental anomalies and/or acute deficits in insulin secretion. To distinguish between developmental versus acute effects of TrkA signaling, we employed a chemical-genetic approach to inducibly silence TrkA receptor tyrosine kinase activity in mature mice at 2 months of age. TrkAF592A knock-in mice express receptors with a mutated ATP binding pocket that can be selectively, rapidly and reversibly inhibited by a small molecule membrane-permeable inhibitor, 1NMPP1 (Chen et al., 2005) **(Fig. 3-2A)**. 1NMPP1 treatment markedly attenuated TrkA phosphorylation in islets isolated from TrkAF592A mice, but had no effect in wild-type islets **(Fig. 3-2B,C)**. Importantly, acutely disrupting TrkA activity in vivo by injecting 1NMPP1 (20 ng/g body weight, intra-peritoneally), 20 minutes prior to a glucose challenge, elicited glucose intolerance in adult

TrkAF592A mice **(Fig. 3-2D)**, similar to the effects observed in *Pdx1-Cre;TrkA$^{f/f}$* mice. The area under the curve (AUC) values of plasma glucose was significantly higher with 1NMPP1 treatment compared to vehicle (DMSO) injection **(Fig. 3-2E)**. Furthermore, TrkAF592A mice treated with 1NMPP1 showed attenuated insulin secretion in the first phase of a glucose challenge, as well as dampened insulin levels in the sustained second phase **(Fig. 3-2F)**. Consistently, 1NMPP1 treatment also significantly decreased GSIS in TrkAF592A islets in static insulin secretion assays **(Fig 3-2G)**.

There were no differences in total islet insulin content and insulin sensitivity, assessed by insulin tolerance assays, with 1NMPP1 versus vehicle injections in TrkAF592A mice **(Fig. 3-2H,I)**. Thus, glucose intolerance in 1NMPP1-treated mice likely does not arise from defects in insulin biosynthesis and insulin responsiveness. Notably, 1NMPP1 treatment did not alter glucose tolerance or insulin secretion in wild-type animals **(Fig. 3-3A-C)**, highlighting that the effects of 1NMPP1 are specific in the context of the TrkAF592A mutation. Together, these results indicate that TrkA signaling acutely regulates glucose homeostasis and insulin secretion, in a manner independent of developmental effects.

Figure 3-1: Conditional deletion of TrkA in the pancreas impairs glucose tolerance and insulin secretion

(A,B) Biotinylated NGF binding is undetectable in *Pdx1-Cre;TrkA$^{f/f}$* islets (outlined in red dashed line) compared to control *TrkA$^{f/f}$* islets. Scale bar, 100 µm for **(A,B)**. Representative images for **(A,B)** are shown from at least 3 animals per genotype that were analyzed. **(C,D)** *Pdx1-Cre;TrkA$^{f/f}$* mice have normal fasted blood glucose levels, but are slightly hyperglycemic in the fed state. Values are the mean ± SEM for n=13 control, 14 mutant mice for **(C)** and n=19 control, 15 mutant mice for **(D)**. *p<0.05, two-tailed *t*-test. **(E,F)** *Pdx1-Cre;TrkA$^{f/f}$* mice have impaired glucose tolerance and reduced circulating insulin compared to *TrkA$^{f/f}$* litter-mates. Mice were injected with glucose (2g/kg body weight) delivered intra-peritoneally (i.p.), after an overnight (16 hour) fast. Values are the mean ± SEM for n=13 control, 14 mutant mice for glucose tolerance tests, and n=4 mice per genotype for insulin secretion tests. *p<0.05, **p < 0.01, ***p<0.001, two-tailed *t*-test with Welch's correction for unequal variance where necessary. Philip Borden contributed data to **(C)** n=8 control and n=14 mutant mice, **(D)** n=17 control and n=8 mutant mice, **(E)** n =7 control mice and n= 10 mutant mice.

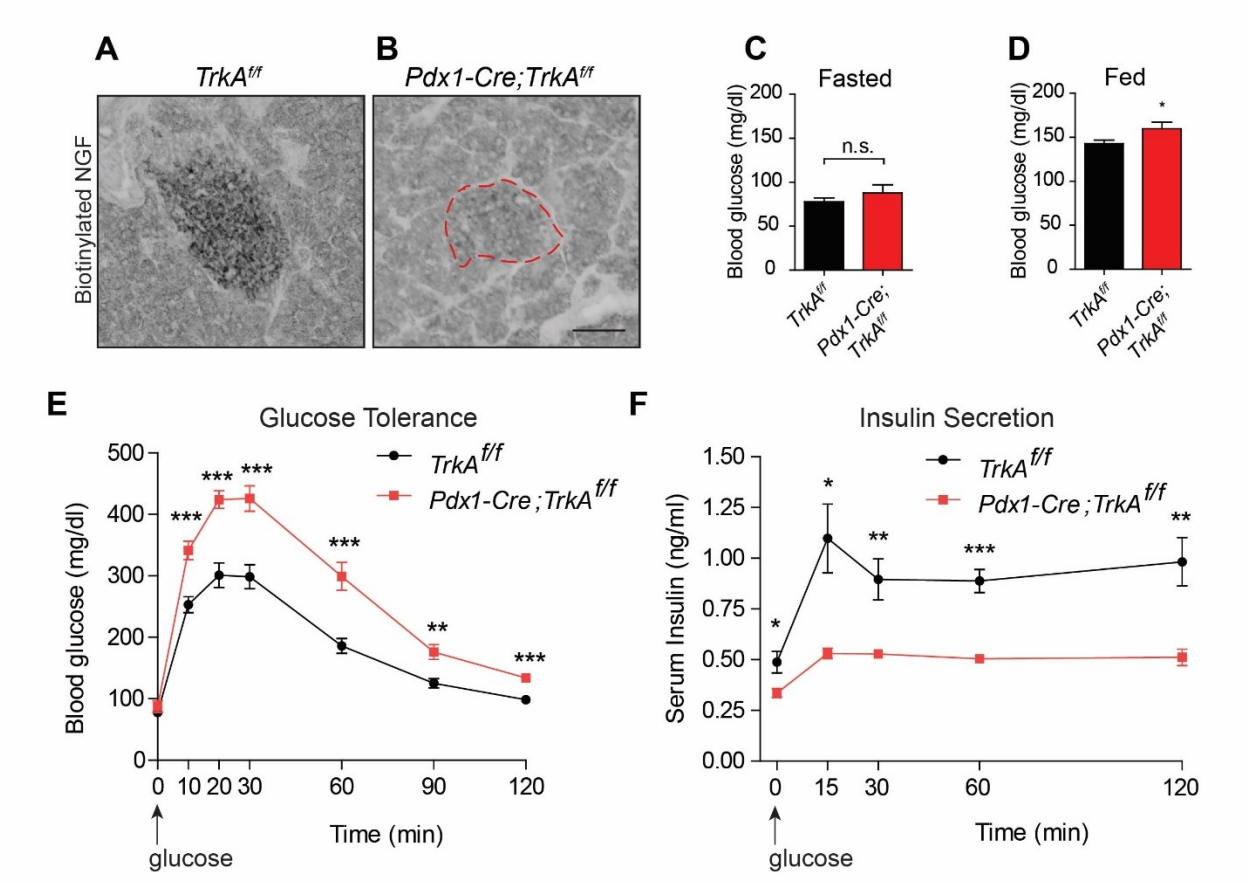

A
TrkA f/f
B
Pdx1-Cre;TrkA f/f
Biotinylated NGF
C
Fasted
Blood glucose (mg/dl)
200
150
100
50
0
n.s.
TrkA f/f
Pdx1-Cre; TrkA f/f
D
Fed
Blood glucose (mg/dl)
200
150
100
50
0
*
TrkA f/f
Pdx1-Cre; TrkA f/f
E
Glucose Tolerance
Blood glucose (mg/dl)
500
400
300
200
100
0
TrkA f/f
Pdx1-Cre ;TrkA f/f
***
***
***
***
**
***
0 10 20 30 60 90 120
Time (min)
glucose
F
Insulin Secretion
Serum Insulin (ng/ml)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
TrkA f/f
Pdx1-Cre ;TrkA f/f
*
*
**
***
**
0 15 30 60 120
Time (min)
glucose

Figure 3-2: Acute disruption of TrkA activity impairs glucose tolerance and insulin secretion

(A) A chemical genetic strategy to inducibly silence TrkA kinase activity with the small molecule inhibitor, 1NMPP1, in *TrkA*^{F592A} knock-in mice carrying a mutation in the ATP binding pocket. (**B,C)** 1NMPP1 (20μM) treatment for 20 minutes decreased TrkA phosphorylation in *TrkA*^{F592A} islets, but had no effect on wild-type islets. Phospho-TrkA immunoblots were stripped and re-probed for TrkA for normalization. Densitometric quantification of phospho-TrkA levels normalized to total TrkA levels. Values are expressed relative to vehicle-treated *TrkA*^{F592A} or wild-type islets. Results are means ± SEM from 3 independent experiments. *p<0.05, *t*-test. (**D**) Acute inactivation of TrkA signaling by 1NMPP1 injection (20ng/g body weight), 20 minutes prior to a glucose challenge, impaired glucose tolerance in *TrkA*^{F592A} mice, compared to vehicle (DMSO) injection. Values are n=6 vehicle (DMSO)- and n=8 1NMPP1-injected *TrkA*^{F592A} mice. *p<0.05, **p<0.01, *t*-test **(E)** Area under the curve (AUC) calculated for glucose tolerance test. *p<0.05, *t*-test. **(F)** *In vivo* glucose-stimulated insulin secretion is attenuated by 1NMPP1 injection 20 minutes prior to the glucose challenge in *TrkA*^{F592A} mice. Values are n=5 mice each for vehicle (DMSO)- and 1NMPP1-injections. *p<0.05, ***p<0.001, *t*-test. **(G)** Glucose stimulated insulin secretion is attenuated by 1NMPP1 pre-treatment (20μM, 20 minutes) in isolated *TrkA*^{F592A} islets. Values are means ± SEM from n=6 independent experiments. Islets were isolated from a total of 4-5 mice per experiment. ***p<0.001, two-way ANOVA with Bonferroni post-hoc test. **(H)** Total islet insulin content is normal with 1NMPP1 treatment (20μM, 20 minutes). Values are means ± SEM from n=5 independent experiments. n.s. not significant, *t*-test. **(I)** Normal insulin sensitivity in

TrkA^{F592A} mice treated with 1NMPP1 (20μM). A significant decrease in blood glucose levels was seen in both 1NMPP1- and vehicle-injected *TrkA*^{F592A} mice, 15 minutes after insulin injection compared to t=0. Mice were treated with 0.75U/kg of insulin (i.p.), and blood glucose measured from tail blood at the indicated times post-injection. Values are mean ± SEM from n=5 mice each for 1NMPP1 and vehicle injections. **p<0.01,***p<0.001, *t*-test.

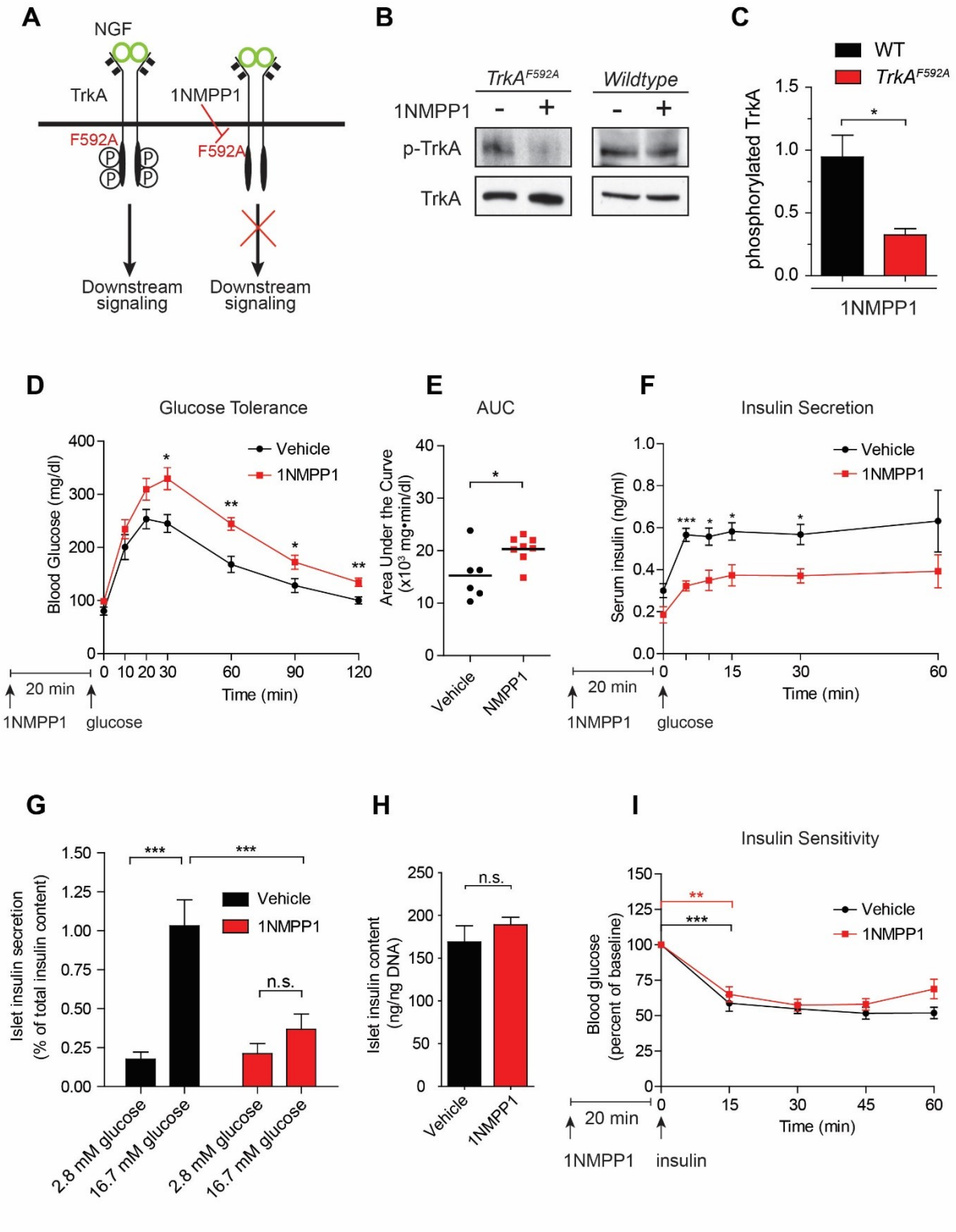
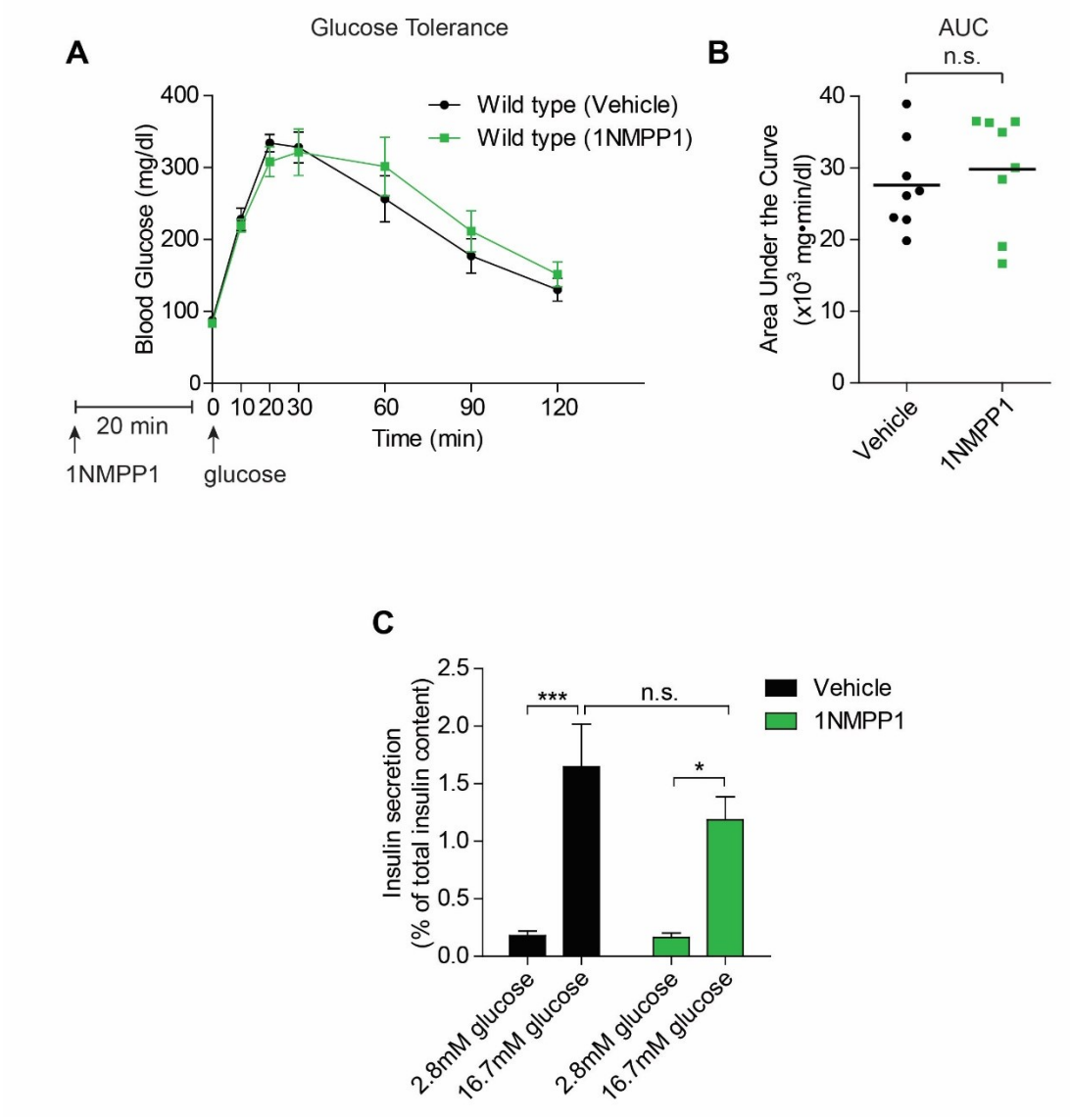

A
NGF
TrkA
1NMPP1
F592A
F592A
Downstream signaling
Downstream signaling
B
TrkA F592A
Wildtype
1NMPP1 - + - +
p-TrkA
TrkA
C
WT
TrkA F592A
phosphorylated TrkA
1NMPP1
D
Glucose Tolerance
Vehicle
1NMPP1
Blood Glucose (mg/dl)
Time (min)
20 min
1NMPP1
glucose
E
AUC
Area Under the Curve (x10³ mg•min/dl)
Vehicle
NMPP1
F
Insulin Secretion
Vehicle
1NMPP1
Serum insulin (ng/ml)
Time (min)
20 min
1NMPP1
glucose
G
Islet insulin secretion (% of total insulin content)
Vehicle
1NMPP1
n.s.
2.8 mM glucose
16.7 mM glucose
2.8 mM glucose
16.7 mM glucose
H
Islet insulin content (ng/ng DNA)
n.s.
Vehicle
1NMPP1
I
Insulin Sensitivity
Vehicle
1NMPP1
Blood glucose (percent of baseline)
Time (min)
20 min
1NMPP1
insulin

Figure 3-3: 1NMPP1 treatment does not influence glucose tolerance or insulin secretion in wild-type mice

(A) Glucose tolerance tests for wild-type mice injected with 1NMPP1 or vehicle (DMSO) 20 minutes prior to an intra-peritoneal glucose challenge. **(B)** Area under the curve (AUC) analysis for glucose tolerance tests in **(A)**. Values are means ± SEM for n=8 animals each for vehicle and 1NMPP1 injections. **(C)** Normal glucose-stimulated insulin secretion in wild-type islets treated with 1NMPP1. Values are means ± SEM from n=4 independent experiments, $*p<0.05$, $***p<0.001$, n.s. not significant, two-way ANOVA with Bonferroni post-test.


A
Glucose Tolerance
Wild type (Vehicle)
Wild type (1NMPP1)
Blood Glucose (mg/dl)
400
300
200
100
0
0 10 20 30 60 90 120
Time (min)
20 min
1NMPP1
glucose
B
AUC
n.s.
Area Under the Curve
(x10^3 mg•min/dl)
40
30
20
10
0
Vehicle
1NMPP1
C
Insulin secretion
(% of total insulin content)
2.5
2.0
1.5
1.0
0.5
0.0
***
n.s.
*
Vehicle
1NMPP1
2.8mM glucose
16.7mM glucose
2.8mM glucose
16.7mM glucose

DISCUSSION

Previous work on neurotrophin regulation of metabolism has primarily focused on central hypothalamic circuits that control appetite and energy balance (Fargali et al., 2012, Rios, 2013). Despite the expression of neurotrophins and their receptors in peripheral metabolic tissues, little is known about peripheral mechanisms by which neurotrophins influence metabolism. Recently, p75 receptors in adipocytes were found to regulate energy expenditure and obesity, although this effect was independent of the p75 extracellular domain and neurotrophin binding (Baeza-Raja et al., 2016). Together, with our data from conditional NGF deletion from the vasculature, our findings in mice with a pancreas-specific deletion of TrkA demonstrate a direct role for NGF/TrkA signaling in controlling insulin secretion and blood glucose homeostasis.

Prior studies focused on the effects of prolonged exposure to NGF on β-cell morphology, viability, and function. Here, we demonstrate that acute TrkA activity, during a glucose challenge, is important for GSIS in β-cells. Pancreas-specific deletion of TrkA was also found to result in reduced islet size, thus it is likely that loss of TrkA signaling has consequences for β-cell proliferation or survival *in vivo.* Additional analysis of *Pdx-Cre;TrkA*$^{f/f}$ mice during development will be informative in elucidating a developmental role for neurotrophin signaling in β-cells. It is important to note that our work is of clinical relevance given that *TrkA* mutations have been linked to impaired glucose-stimulated insulin secretion in humans (Schreiber et al., 2005), and suggest the utility of neurotrophins and small molecule receptor agonists in the treatment of type 2 diabetes.

METHODS

Mice

All procedures relating to animal care and treatment conformed to Johns Hopkins University Animal Care and Use Committee (ACUC) and NIH guidelines. Mice were housed in a standard 12:12 light-dark cycle. Mice were maintained on a *C57BL/6* background, or mixed *C57BL/6* and *129P*, or *C57BL/6* and *FVB* backgrounds. Both sexes were used for analyses at 1-2 months of age, unless stated otherwise in the figure legends. $TrkA^{F592A}$ ($TrkA^{f/f}$) mice were obtained from the Jackson Laboratory (Stock No: 022362), and *Pdx1-Cre* mice ($Tg(Pdx1\text{-}Cre^{Tuv})$, strain no: 01XL5) mice were obtained from NCI Frederick mouse repository.

Antibodies

Primary antibodies for western blots include rabbit anti-Phospho-TrkA (Tyr785)/TrkB (Tyr816) (1:1000, Cell Signaling C67C8) and rabbit anti-TrkA (1:1000, Millipore 06-574)

Ligand binding

Pancreatic NGF receptors were labeled by incubating 10 μm thick mouse tissue sections with biotinylated NGF (200ng/ml) for 1 hour at room temperature. Biotinylated NGF was prepared according to the manufacturer's instructions using EZ-Link™ Micro Sulfo-NHS-Biotinylation kit (ThermoFisher Scientific, 21925). Following incubation with biotinylated NGF, tissue sections were washed and immunostained for insulin as described below. Biotinylated NGF was detected using Alexa-546-conjugated streptavidin (1:200,

Invitrogen, S11225). Sections were imaged using a Zeiss AxioImager M1 microscope equipped with an Axiocam HRc camera.

Islet isolations

Islets were isolated as previously described (Wollheim et al., 1990). Briefly, pancreata collected from 1-2-month old mice were distended using collagenase (Collagenase P, 0.375 mg/ml, Roche) dissolved in Hank's Balanced Salt Solution (HBSS, Mediatech), and digested at 37°C. Digested pancreata were washed with HBSS, and subjected to discontinuous density gradient centrifugation using varying histopaque densities (Sigma-Aldrich). The islet layer was collected, washed with HBSS, and islets handpicked under an inverted microscope for subsequent analyses.

Immunoblotting

For detection of phosphorylated TrkA, isolated islets from *TrkAF592A* mice were allowed to recover overnight in RPMI 1640 medium containing 5% fetal bovine serum (FBS) and 5 U/l penicillin/streptomycin. Islets were incubated with 1NMPP1 (20 μM) or vehicle (DMSO) dissolved in RPMI 1640 medium for 20 min, and then lysed in boiling laemmli buffer. For assessing glucose effects on TrkA phosphorylation, islets were pre-incubated in Krebs-Ringer HEPES buffer (KRHB) containing low (2.8 mM) glucose for 1 hour, and then either left in 2.8 mM glucose or stimulated with 16.7 mM glucose in KRHB buffer for 15 minutes, and lysates prepared in boiling laemmli buffer. Lysates were subjected to immunoblotting with rabbit anti-Phospho-TrkA (Tyr785)/TrkB (Tyr816), and membranes were later re-probed for rabbit anti-TrkA. All immunoblots were visualized with ECL Plus

Detection Reagent (Thermo Scientific, 32132) and scanned with a Typhoon 9410 Variable Mode Imager (GE Healthcare). Densitometric analysis of bands was performed using ImageJ software.

In vivo analyses of glucose tolerance, insulin tolerance, and circulating insulin

For glucose tolerance tests, 1-2 month-old mice were fasted overnight, with a blood glucose reading the evening before the assay serving as a fed blood glucose measurement. The next morning, mice were injected with glucose (2g/kg, i.p). Blood glucose measurements were made from tail blood using a OneTouch Ultra glucometer at the indicated times (Gu et al., 2010). For acute treatments with 1NMPP1, mice received i.p. injections with 20ng/g 1NMPP1 or DMSO, 20 minutes prior to glucose administration.

For *in vivo* insulin and NGF secretion assays, mice were fasted overnight before being injected with glucose (3 g/kg, i.p.). Blood was collected from the tail at the times indicated, spun down, and the resulting plasma fractions subjected to insulin ELISA (Crystal Chem, 90080). Reactions were assessed using a Tecan infinite 200 plate reader.

For insulin sensitivity tests, mice were separated into individual cages with food the evening prior to the assay. The next morning, mice were treated with 0.75 U/kg of insulin (Novolin-R; Novo Nordisk), and blood glucose measurements were made from tail blood at the indicated times (Bruning et al., 1997).

In vitro GSIS assays

For insulin secretion assays in isolated islets, harvested islets pooled from 4-5 mice were allowed to recover overnight in RPMI 1640 media containing 5% fetal bovine serum (FBS) and 5 U/l penicillin/streptomycin. Islets were washed in Krebs-Ringer HEPES buffer (KRHB) containing low (2.8 mM) glucose and allowed to stabilize for 1 hour. Islets were then pre-incubated with vehicle (DMSO) or 1NMPP1 (20μM) for 20 minutes. Groups of 5-10 islets were then handpicked into 24-well dishes and incubated in low (2.8 mM) or high glucose (16.7 mM) in KRHB buffer in the indicated conditions (vehicle or 1NMPP1) for another 30 minutes. Supernatant fractions were removed, the islets were lysed in acid ethanol, followed by insulin ELISA (Crystal Chem) to determine the insulin concentrations in both supernatant and islet fractions.

For insulin content measurements, $TrkA^{F592A}$ islets incubated in low glucose-KRHB media were treated with 1NMPP1 (20μM) or DMSO for 20 minutes. Islets were lysed with acid ethanol, and insulin content was determined by ELISA and normalized to DNA content, measured from the same lysates using a PicoGreen kit (Invitrogen). Absorbance readings were taken using a Tecan infinite 200 plate reader.

CHAPTER 4: TRKA SIGNALING IS REQUIRED FOR GLUCOSE STIMULATED INSULIN GRANULE RECRUITMENT AND ACTIN REMODELING IN β-CELLS

Portions of this chapter were previously submitted for publication at Developmental Cell: Jessica Houtz, Philip Borden, Alexis Ceasrine, and Rejji Kuruvilla (2016). *Neurotrophin signaling is required for glucose-induced insulin secretion.*

INTRODUCTION

Actin remodeling regulates GSIS

The disparate roles of actin in regulated exocytosis have been well-documented with evidence to support both negative and positive effects in vesicle secretion (Porat-Shliom et al., 2013, Kalwat and Thurmond, 2013, Varadi et al., 2005, Schuh, 2011). On the one hand, F-actin tracks are needed for mobilizing vesicles from deeper reserve pools to the plasma membrane (Varadi et al., 2005, Schuh, 2011). On the other hand, cortical F-actin acts as a physical barrier to limit exocytosis in diverse cell types including endocrine, chromaffin, immune cells and neurons (Porat-Shliom et al., 2013, Kalwat and Thurmond, 2013). In β-cells, the barrier function of the cytoskeleton has been proposed to be critical for maintaining low levels of insulin release under basal conditions (Kalwat and Thurmond, 2013). Importantly, the presence of a dense cytoskeletal meshwork at the cell periphery presents a layer of regulation for controlled insulin release under elevated glucose (Zhu et al., 2015).

Each individual β-cell contains approximately 10,000 secretory granules of insulin but only a fraction (several hundreds) are released at a time in response to high glucose, emphasizing the precise regulation of release probability of insulin granules (Rorsman and Renstrom, 2003). The relevance of actin dynamics in glucose homeostasis is exemplified by genetic studies in mice where deletion of Rac1, or its effector, p21-activated kinase (PAK), elicits glucose intolerance and diminished insulin secretion (Asahara et al., 2013, Wang et al., 2011). Notably, a 10-fold increase in total cellular actin has been observed in islets from type 2 diabetes patients (Ostenson et al., 2006). Although a number of actin

regulators of glucose-stimulated insulin secretion including Rac1, Cdc42, PAK, Focal Adhesion Kinase (FAK), cofilin, and gelsolin, have been identified in β-cells (Thurmond et al., 2003, Kalwat and Thurmond, 2013), the events upstream of these actin-modulatory proteins have remained unclear.

NGF signaling through TrkA regulates cytoskeletal dynamics in sympathetic neurons

In neurons, the cytoskeleton is important for determining the structure of axonal and dendritic arborizations. Neurotrophins have well-established roles in influencing neurite formation, axon outgrowth and branching, and dendritic complexity (Gallo and Letourneau, 2000, McAllister et al., 1999, Ruit and Snider, 1991). Additionally, neurotrophin signaling can directly impact levels of cytoskeletal proteins, in particular filamentous actin (F-actin), in growth cones by affecting both protein and mRNA localization (McAllister et al., 1999, Zhang et al., 1999, Wickramasinghe et al., 2008). NGF has also been demonstrated to influence axon growth by stabilizing neurofilaments (Veeranna et al., 1998) and modulating GSK-3β and the plus-end microtubule binding protein, APC, to stabilize microtubules in the axon shaft. Besides directly affecting elements of the cytoskeleton, NGF can stimulate the autocrine production of Wnt5a to indirectly promote axon branching during development (Bodmer et al., 2009).

Studies have shown that TrkA forms signaling endosomes that associate with Rap1 (Wu et al., 2001, York et al., 1998, York et al., 2000) which could provide a platform on which interacting proteins such as RIAM and the Rho family of small GTPases may accumulate to regulate actin dynamics in the growth cone. Numerous lines of evidence suggest that NGF affects cellular processes in axons via modulation of cytoskeletal-

associated protein such as actin depolymerizing factor (ADF) and Rac1, and recent work has highlighted an important role for TrkA endocytosis in mediating local actin depolymerization to enable retrograde transport of NGF signals through the dense F-actin meshwork in axons (Harrington et al., 2011).

Since regulation of actin dynamics is a shared feature of both neurotrophin signaling in neurons and glucose stimulated insulin secretion in β-cells, we hypothesized that TrkA signaling might be affecting insulin secretion through its influence on actin remodeling. In this chapter, we show a previously unappreciated mechanism through which TrkA can acutely influence insulin granule recruitment to the cell surface and subsequent secretion.

RESULTS

TrkA signaling is required for surface-localization of insulin granules

Regulatory control of insulin secretion can occur at the level of glucose uptake and metabolism in β-cells, β-cell membrane depolarization, and insulin granule mobilization and exocytosis (MacDonald et al., 2005). A key question is, which step in this stimulus-secretion coupling pathway is affected by TrkA signaling? Exposure to high extracellular K+ is a well-established experimental strategy to depolarize the β-cell plasma membrane, thus by-passing the need for glucose uptake and metabolism to trigger insulin secretion (Hatlapatka et al., 2009). Elevated potassium chloride (KCl) results in the opening of voltage-dependent Ca^{2+} channels to promote the exocytosis of secretion-ready insulin granules (Hatlapatka et al., 2009). To determine if the requirement for TrkA signaling in insulin secretion was downstream of glucose sensing and metabolism, we assessed the effects of 1NMPP1 on KCl-induced insulin secretion. 1NMPP1-mediated inhibition of TrkA activity abolished insulin secretion induced by high KCl (30mM) in TrkAF592A islets **(Fig. 4-1A)**. These results suggest that TrkA signaling is required in the step(s) of the insulin secretory pathway that is distal to membrane depolarization.

To further probe the requirement for TrkA activity in insulin secretion, we employed ultra-structural analyses. Electron microscopy revealed an increase in insulin granule localization at the β-cell plasma membrane in response to an in vivo glucose challenge compared to the fasted state in TrkAF592A mice **(Fig. 4-1B,C, and F)**. However, 1NMPP1 injection, 20 minutes prior to glucose administration, suppressed the glucose-induced recruitment of insulin granules to the cell periphery, but had no effect on

insulin docking under basal conditions **(Fig. 4-1D,E, and F)**. 1NMPP1-treated β-cells had fewer docked insulin granules (quantified as granules within 50 nm of the plasma membrane) in the presence of high glucose, although total number of insulin granules and their sizes were normal. Since islet insulin content was unaffected by 1NMPP1 treatment **(see Chapter 3 Fig. 3-2H)**, the reduction in surface-localized insulin granules is likely not due to defects in insulin biogenesis. Together, these results suggest that TrkA activity is necessary for glucose-dependent insulin granule positioning at the β-cell surface.

TrkA signaling is required for F-actin remodeling in β-cells

Given that a brief (20 minute) exposure to 1NMPP1 was sufficient to elicit glucose intolerance and attenuate GSIS in TrkAF592A mice, we reasoned that TrkA signaling likely influences insulin secretion by mechanisms independent of gene transcription. Actin rearrangement is a critical acute determinant of GSIS35. Actin microfilaments are organized as a dense meshwork beneath the β-cell plasma membrane that restricts insulin granule access to the docking and fusion machinery (Orci et al., 1972, van Obberghen et al., 1973, Li et al., 1994). Glucose stimulation rapidly promotes filamentous actin (F-actin) remodeling to mobilize insulin granules to the cell periphery (Kalwat and Thurmond, 2013), (Howell and Tyhurst, 1986, Thurmond et al., 2003, Wang and Thurmond, 2009). However, how glucose stimulation regulates actin reorganization has remained unclear.

Remodeling of the actin cytoskeleton is also a key cellular process by which neurotrophins control vesicular trafficking in neurons (Harrington and Ginty, 2013). Thus, we asked whether TrkA signaling promotes actin rearrangements in β-cells, a pre-requisite step in insulin granule positioning at the plasma membrane. Isolated TrkAF592A β-cells

were pre-treated with 1NMPP1 for 20 minutes prior to stimulation with either low (2.8 mM) or high glucose (16.7 mM), and F-actin was visualized using Alexa-546-labeled phalloidin. Under basal conditions (2.8 mM glucose), both vehicle and 1NMPP1-treated β-cells showed a thick F-actin cortical ring **(Fig. 4-2A,C)**. High glucose treatment elicited a striking reduction in F-actin **(Fig. 4-2B,E)**, consistent with previous reports (Nevins and Thurmond, 2003, Cai et al., 2012). However, the glucose-induced dissolution of F-actin was prevented by 1NMPP1-mediated silencing of TrkA signaling **(Fig. 4-2D,E)**. Thus, TrkA activity is required for glucose-triggered actin reorganization in β-cells.

Glucose-mediated actin remodeling in β-cells depends on the activities of several actin-modulatory proteins including the Rac1 GTPase (Kalwat and Thurmond, 2013). In clonal β-cell lines and in islets, elevated glucose stimulates Rac1 activity (Kowluru, 2011). β-cell-specific deletion of Rac1 inhibits F-actin disassembly and impairs glucose tolerance and GSIS in mice (Asahara et al., 2013). Rac1-deficient β-cells also show impaired glucose-dependent recruitment of insulin granules to the β-cell membrane (Asahara et al., 2013), similar to that seen with TrkA inhibition. However, how elevated glucose activates Rac1 remains unclear. Using an ELISA-based immunoassay, we observed that high glucose treatment for 15 minutes significantly increased GTP-bound Rac1 levels (1.8 ± 0.15-fold increase) in TrkAF592A islets, which was suppressed by TrkA inhibition with 1NMPP1 **(Fig. 4-2F)**. These results suggest that TrkA kinase activity is required for glucose-mediated activation of Rac1.

We next reasoned that if indeed a perduring F-actin barrier was a key contributor to decreased GSIS upon TrkA inhibition, then forcing F-actin disassembly should alleviate impaired secretion in 1NMPP1-treated islets. To test this prediction, we assessed insulin

secretion in islets that were co-treated with 1NMPP1 and cytochalasin D, an actin depolymerizing agent. As expected, 1NMPP1 treatment alone attenuated GSIS compared to vehicle (DMSO) in TrkAF592A islets **(Fig. 4-2G)**. Remarkably, a normal insulin secretory response in response to elevated glucose was observed in TrkAF592A islets treated with both 1NMPP1 and cytochalasin D **(Fig. 4-2G)**. Together, these results indicate that TrkA-mediated actin reorganization is critical for glucose-induced insulin secretion.

Figure 4-1: TrkA signaling regulates surface recruitment of insulin granules

(A) TrkA signaling is necessary for KCl-induced insulin secretion. Pre-treatment with 1NMPP1 (20μM, 20 minutes) suppresses insulin secretion in response to high KCl (30 mM, 30 minutes) in isolated *TrkA*^{F592A} islets. Values are mean ± SEM from n=5 independent experiments. **p<0.01, ***p<0.001, n.s. not significant, two-way ANOVA with Bonferroni post-test. **(B-E)** TrkA signaling is necessary for glucose-induced surface localization of insulin granules. Electron microscopic analysis of β-cells shows more docked insulin granules (red arrowheads) in response to a glucose challenge, compared to the fasted state, in *TrkA*^{F592A} mice. 1NMPP1 injection, 20 minutes prior to glucose delivery, decreases the glucose-induced recruitment of insulin granules. 1-2 month-old *TrkA*^{F592A} mice were fasted overnight and then injected with vehicle or 1NMPP1 (20ng/g, i.p.), 20 minutes prior to an *in vivo* glucose challenge (2g/kg, i.p.). At least three islets were selected at random per animal, and representative images of β-cells per islet taken. Insulin granules within 50 nm of the plasma membrane were considered as docked granules. Scale bar, 1 μm. **(F)** Quantification of docked insulin granules per micron of plasma membrane observed in electron microscopy experiments. Values are mean ± SEM from n=3 mice each for vehicle and 1NMPP1 injections, **p<0.01, n.s. not significant, two-way ANOVA with Bonferroni post-test.

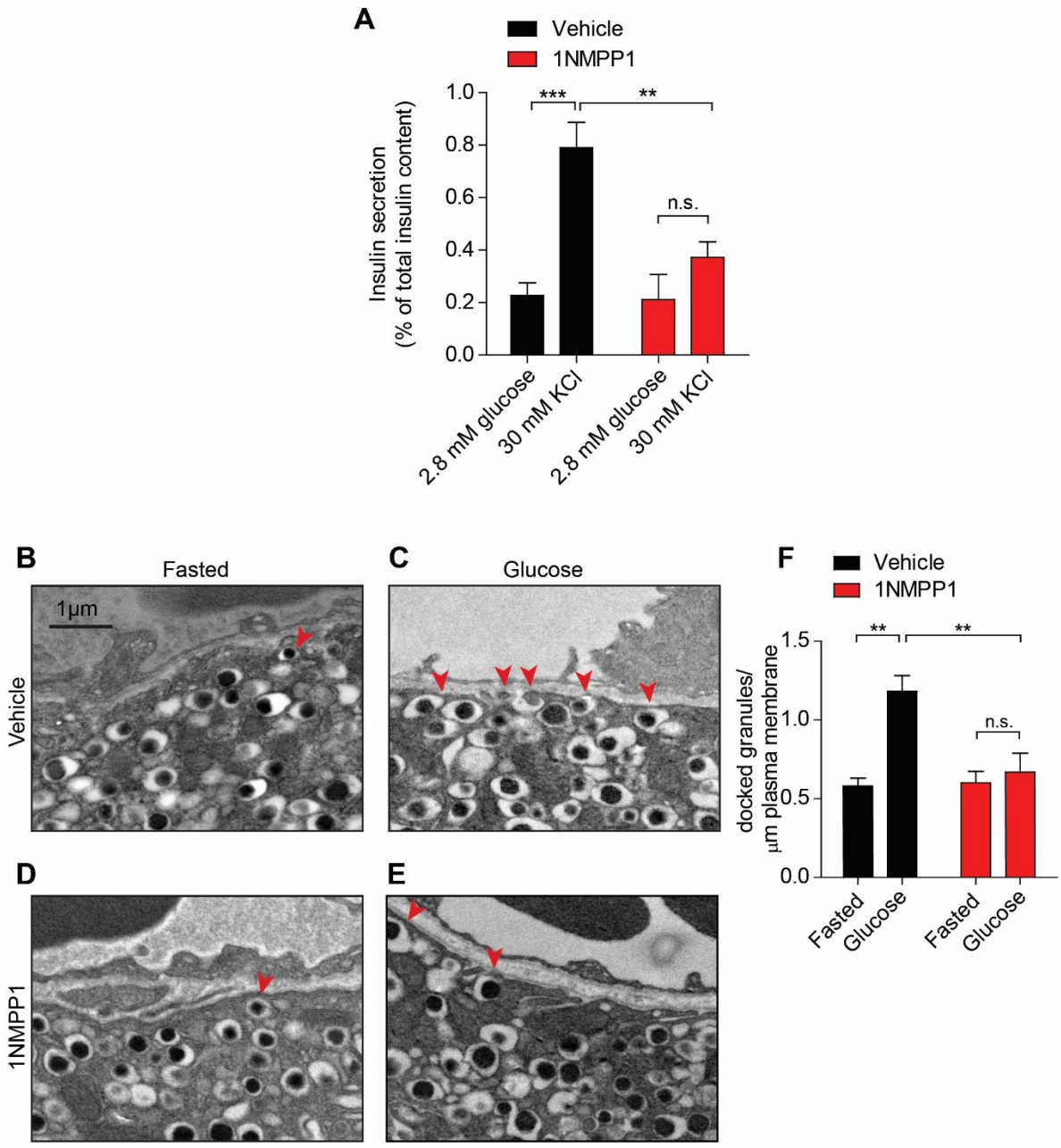
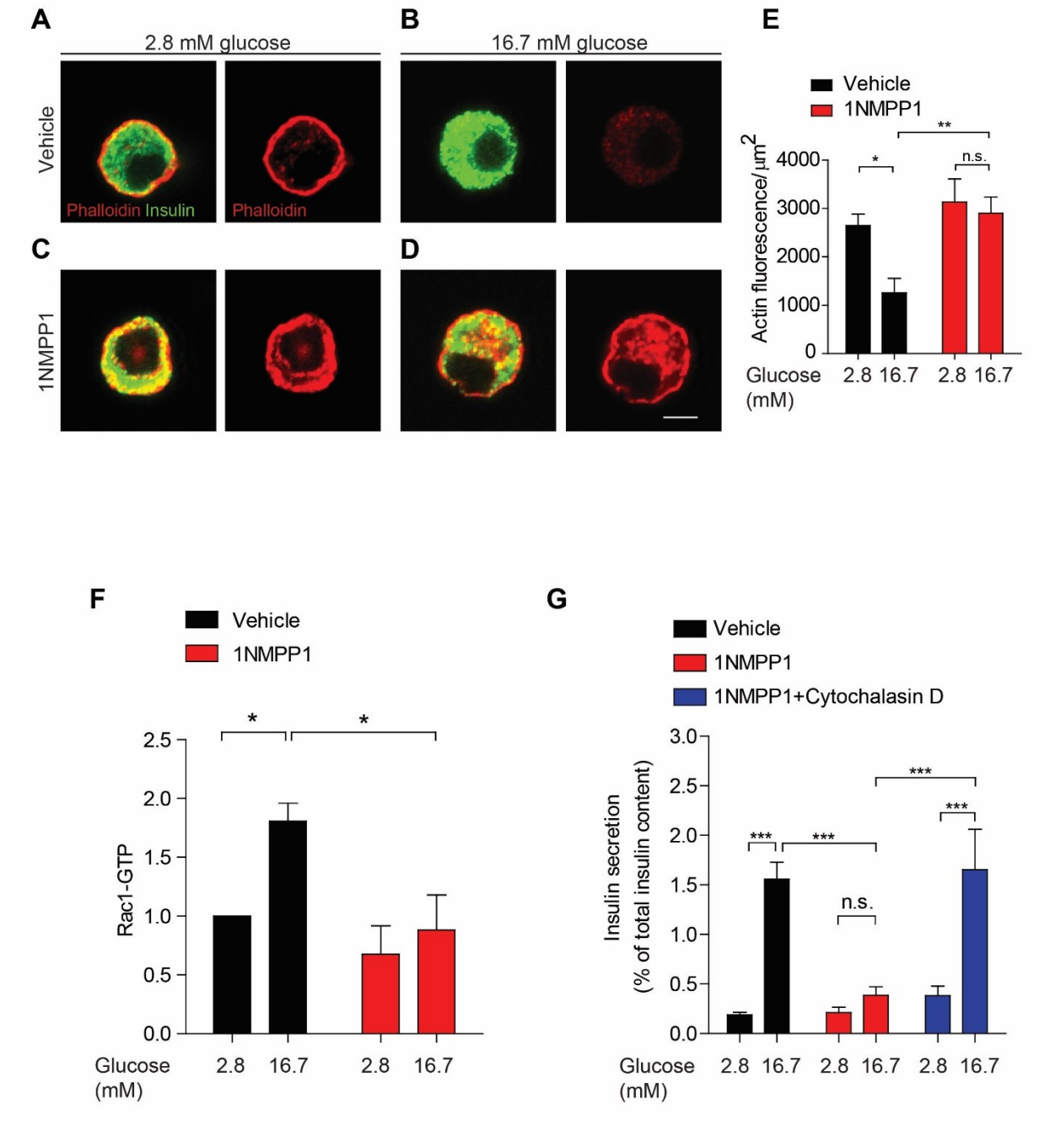

A
Vehicle
1NMPP1
Insulin secretion
(% of total insulin content)
1.0
0.8
0.6
0.4
0.2
0.0
***
**
n.s.
2.8 mM glucose
30 mM KCl
2.8 mM glucose
30 mM KCl
B
Fasted
C
Glucose
1µm
Vehicle
D
E
1NMPP1
F
Vehicle
1NMPP1
docked granules/
µm plasma membrane
1.5
1.0
0.5
0.0
**
**
n.s.
Fasted
Glucose
Fasted
Glucose

Figure 4-2: TrkA activity is required for glucose-stimulated actin remodeling

(A-D) High glucose (16.7 mM) treatment for 30 minutes markedly decreases F-actin levels in primary β-cells from *TrkA*^{F592A} mice, compared to low glucose (2.8 mM) conditions. Glucose-dependent changes in F-actin were prevented by pre-treatment of β-cells with 1NMPP1 (20 μM, 20 minutes). F-actin is marked by Alexa-546-phalloidin (in red) and β-cells in islet cultures were identified by insulin immunostaining (in green). Scale bar, 5μm. **(E)** Average fluorescence intensity for F-actin. Values are mean ± SEM from n=7 independent experiments, 20-25 cells were analyzed per condition per experiment. *p<0.05, **p<0.01, n.s. not significant, two-way ANOVA with Bonferroni post-test. **(F)** Levels of Rac1-GTP are enhanced by elevated glucose treatment (16.7 mM, 15 min) in *TrkA*^{F592A} islets. Pre-incubation of islets with 1NMPP1 for 20 minutes attenuates the glucose-induced increase in Rac1 activity. Values are mean ± SEM from n=5 independent experiments, *p<0.05, two-way ANOVA with Bonferroni post-test. **(G)** Defects in glucose-induced insulin secretion with 1NMPP1 treatment in *TrkA*^{F592A} islets were fully rescued by cytochalasin D, an actin depolymerizing agent. *TrkA*^{F592A} islets were pre-treated with vehicle, 1NMPP1 (20 μM), or 1NMPP1 (20 μM) plus cytochalasin D (25 μM) for 20 minutes, and then stimulated with 16.7 mM glucose or maintained in 2.8 mM glucose for 30 minutes, followed by measurements of insulin content in supernatants and islet lysates. Values are mean ± SEM from n=6 independent experiments. ***p<0.001, n.s. not significant, two-way ANOVA with Bonferroni post-test.


A
2.8 mM glucose
B
16.7 mM glucose
Vehicle
Phalloidin Insulin
Phalloidin
C
1NMPP1
D
E
Vehicle
1NMPP1
Actin fluorescence/μm2
4000
3000
2000
1000
0
*
**
n.s.
Glucose (mM)
2.8 16.7 2.8 16.7
F
Vehicle
1NMPP1
Rac1-GTP
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Glucose (mM)
2.8 16.7 2.8 16.7
G
Vehicle
1NMPP1
1NMPP1+Cytochalasin D
Insulin secretion (% of total insulin content)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
***
***
***
n.s.
Glucose (mM)
2.8 16.7 2.8 16.7 2.8 16.7

DISCUSSION

Biphasic insulin secretion involves an immediate, first phase in which a limited number of pre-docked and primed insulin granules rapidly fuse with the plasma membrane in response to calcium influx, and a sustained second phase that requires insulin granule recruitment (Henquin et al., 2003, Rorsman and Renstrom, 2003). Recent work has demonstrated that elements of the cytoskeleton are dynamically regulated to ensure that insulin secretion remains low under basal insulin secretion and remains elevated as long as glucose levels are high (Kalwat and Thurmond, 2013). We identified a crucial role for TrkA activity, downstream of glucose sensing and metabolism, in regulating β-cell actin dynamics to allow insulin granule recruitment to the plasma membrane.

Deficits in insulin secretion induced by forced β-cell depolarization, similar to our own observations in islets stimulated with KCl while TrkA activity was inhibited **(Fig. 4-1A)**, could indicate an impairment of voltage-gated calcium channels, and there is evidence to support NGF mediated increases in calcium currents in β-cells (Rosenbaum et al., 2002). We do not rule out the possibility that TrkA may influence calcium dynamics in β-cells, however our data supports a significant role for TrkA activity in regulating insulin secretion through actin remodeling, since actin depolymerization was able to rescue insulin secretion back to normal levels **(Fig. 4-2G)**.

Interestingly, some actin remodelers, such as gelsolin (Kalwat et al., 2012) and scinderin (Bruun et al., 2000), are thought to regulate actin dynamics downstream of calcium in β-cells. Conversely, actin depolymerization has been implicated in the regulation of calcium entry via the endoplasmic reticulum (ER) (Patterson et al., 1999, Rosado and Sage, 2000),

which is also at play during the cephalic phase of insulin secretion (Ahrén et al., 2006), and could operate downstream of TrkA signaling via activation of PLCγ. Cortical actin is known to displace endoplasmic reticulum (ER) that acts as a reservoir of intracellular calcium, and cycles of actin dypolymerization and cytosolic calcium are known to regulate pulsatile vesicle secretion (Wollman and Meyer, 2012). There is also data supporting a role for actin deploymerization in generating Ca2+ mobilization signals during GSIS in β-cells (Shawl et al., 2012). Given that β-cell edges have been shown to be enriched in filamentous actin, and are sites of the greatest increases in $[Ca^{2+}]_i$ (Geron et al., 2015), it is possible that TrkA-mediated actin remodeling contributes to the pulsatile nature of Ca^{2+} dynamics and insulin secretion in β-cells.

Impairments in KCl-induced insulin secretion are also typically associated with reduced fusion of pre-docked insulin granules. Our data indicate that inhibition of TrkA activity reduces insulin secretion upon depolarization without influencing the ability of granules to dock to the membrane in basal conditions. There is data to support the contribution of "restless newcomer" granules that fuse directly with the plasma membrane without docking (Shibasaki, T., et al., PNAS 2007, Predersen, MG., Sherman, A., PNAS 2009). Thus, it is possible that TrkA activity could contribute to movement of granules during the first phase of insulin secretion, and inhibition of TrkA activity may influence first phase insulin secretion independently of regulating insulin granule docking. In support of this notion, patients with mutations in the *TrkA* gene have congenital insensitivity to pain and anhidrosis (CIPA) (Indo et al., 1996) and have been characterized to have deficits in first phase insulin secretion (Schreiber et al., 2005). Elucidating the mechanistic regulation behind these different pools of insulin granules in the first phase response could

be accomplished with recent advances in TIRF microscopy and reporters for insulin granules (Seino et al., 2011).

METHODS

Mice

All procedures relating to animal care and treatment conformed to Johns Hopkins University Animal Care and Use Committee (ACUC) and NIH guidelines. Mice were housed in a standard 12:12 light-dark cycle. Mice were maintained on a *C57BL/6* background, or mixed *C57BL/6* and *129P*, or *C57BL/6* and *FVB* backgrounds. Both sexes were used for analyses at 1-2 months of age, unless stated otherwise in the figure legends. $TrkA^{F592A}$ ($TrkA^{f/f}$) mice were obtained from the Jackson Laboratory (Stock No: 022362), 01XL5).

Antibodies

Primary antibodies for immunofluorescence include guinea pig anti-insulin (1:300, abcam ab7842). Fluorescent secondary antibodies were Alexafluor-350, -488,-546, or -647-conjugated and used at 1:200 (Invitrogen)

Islet isolations

Islets were isolated as previously described (Wollheim et al., 1990). Briefly, pancreata collected from 1-2 month old mice were distended using collagenase (Collagenase P, 0.375 mg/ml, Roche) dissolved in Hank's Balanced Salt Solution (HBSS, Mediatech), and digested at 37°C. Digested pancreata were washed with HBSS, and subjected to discontinuous density gradient centrifugation using varying histopaque densities (Sigma-

Aldrich). The islet layer was collected, washed with HBSS, and islets handpicked under an inverted microscope for subsequent analyses.

Electron microscopy

1-2 month old *TrkA*^{F592A} mice were separated into individual cages and fasted overnight, and subjected to either 1NMPP1 (20 ng/g body weight, Cayman Chemical) or DMSO injections administered intra-peritoneally (i.p.). After 20 minutes, pancreata were immediately collected for control conditions, or mice were injected with glucose (Sigma Aldrich, 2 g/kg body weight, i.p). 15 minutes after glucose administration, minced, and fixed in buffer containing 3% formaldehyde, 1.5% glutaraldehyde, 5mM $CaCl_2$, 2.5% sucrose, and 0.1M sodium cacodylate for 1hr at room temperature. Tissues were post-fixed in 1% Palade's OsO_4 for 1hr on ice, followed by incubation in Kellenberger's uranyl acetate overnight at room temperature. After dehydration through graded alcohols, tissues were embedded in Epon, and ultrathin (~90 nm) sections were collected onto EM grids. Grids were then imaged using an FEI Tecnai-12 TWIN transmission electron microscope operating at 100 kV. For each image, the number of granules within 50 nm of the plasma membrane was determined using ImageJ software. Results were expressed as average number of docked insulin granules per μm of the plasma membrane, determined for 3-4 islets per animal, and 3 animals per condition.

***In vitro* GSIS assays**

For insulin secretion assays in isolated islets, harvested islets pooled from 4-5 mice were allowed to recover overnight in RPMI 1640 media containing 10% fetal bovine serum

(FBS) and 5 U/l penicillin/streptomycin. Groups of 5-10 islets of similar size were handpicked into 24-well dishes, washed in Krebs-Ringer HEPES buffer (KRHB) containing low (2.8 mM) glucose and allowed to stabilize for 1 hour. Islets were then pre-incubated with vehicle (DMSO), 1NMPP1 (20μM), or 1NMPP1 (20 μM) plus cytochalasin D (25μM, Cayman Chemical) for 20 minutes. Groups of 5-10 islets were then incubated in low (2.8 mM) or high glucose (16.7 mM), or KCl (30 mM, Sigma-Aldrich) in KRHB buffer in the indicated conditions (Vehicle, 1NMPP1, or 1NMPP1 plus cytochalasin) for another 30 minutes. Supernatant fractions were removed, the islets were lysed in acid ethanol, followed by insulin ELISA (Crystal Chem) to determine the insulin concentrations in both supernatant and islet fractions.

Phalloidin staining

Islets were isolated from adult 1-2 month old *TrkA*^{F592A} mice and dissociated into single cells by gentle trituration in HBSS containing 4 mM EDTA. Cells were resuspended in RPMI 1640 media containing 5% FBS and 5 U/l penicillin/streptomycin, and plated on glass coverslips coated with poly-D lysine. After 24 hours, islet cultures were treated with 1NMPP1 (20 μM) or DMSO for 20 minutes, followed by incubation in low (2.8 mM) or high (16.7 mM) glucose for another 30 minutes. Cells were fixed in 4% PFA and stained with Alexa546–conjugated phalloidin (Invitrogen). β-cells were identified by immunostaining for insulin. Images representing 1μm optical sections were acquired using a Zeiss LSM 510 confocal microscope. Average fluorescence intensities of Alexa-546 per μm^2 in individual β-cells were measured using ImageJ software (NIH). 20-25 cells were analyzed per condition per experiment.

To assess the effects of endocytosis-defective TrkA receptors on F-actin, isolated β-cells from *TrkA*^{F592A} mice were incubated with adenoviruses expressing either FLAG-TrkA^{Y794F} or control FLAG-TrkA receptors, and treated with doxycycline (100 ng/ml) in RPMI 1640 media containing 5% FBS and 5 U/l penicillin/streptomycin for 24 hours. β-cells were then incubated in 1NMPP1 (20μM) for 20 minutes to silence endogenous TrkA receptors, and then fixed and labeled with Alexa546–phalloidin as described above.

Rac1 activity

GTP-bound Rac1 levels in islet lysates were measured using Rac1 G-protein linked immunosorbent assay (G-LISA) (Cytoskeleton Inc., Cat. # BK126). Islets were treated with 1NMPP1 (20 μM) or DMSO for 20 minutes, followed by incubation in low (2.8 mM) or high (16.7 mM) glucose for another 15 minutes. Islet protein concentrations were measured using Precision Red Advanced Protein Assay (Cytoskeleton Inc., Cat. # ADV02-A), and normalized prior to processing the samples for the Rac1 G-LISA assays.

CHAPTER 5: FUNCTIONAL TRKA SIGNALING ENDOSOMES IN β-CELLS

Portions of this chapter were previously submitted for publication at Developmental Cell: Jessica Houtz, Philip Borden, Alexis Ceasrine, and Rejji Kuruvilla (2016). *Neurotrophin signaling is required for glucose-induced insulin secretion.*

INTRODUCTION

TrkA signaling mechanisms

TrkA belongs to the family of high affinity neurotrophin receptor tyrosine kinases, and as such activates well characterized downstream signaling pathways including MAPK, PI3K, and PLC gamma (PLCγ) to mediate complex cell processes (Chao, 2003). Canonical receptor tyrosine kinase signaling begins with ligand binding at the extracellular domain of the receptor and subsequent dimerization, which leads to auto-phosphorylation of the intracellular kinase domain. Intracellular activation of the receptor results in phosphorylation at highly conserved tyrosine residues that recruit and activate cytoplasmic signaling effectors. Specifically, phosphorylation of tyrosine 499 (Y499) in TrkA is known to be required for recruitment and activation of the adaptor protein, Shc that leads to the activation of the MAPK and PI3K pathways (Segal et al., 1996, Stephens et al., 1994), phosphorylation of tyrosine 760 (Y760) specifically recruits the p85 catalytic subunit of PI3K (Obermeier et al., 1993), and tyrosine 794 (Y794) is required for PLCγ activation by TrkA.

While the immediate and local effects of NGF on axon growth and branching are fairly easy to envision, NGF signaling through TrkA at growth cones is also propagated back to the cell body to regulate synaptogenesis and transcriptional changes (Huang and Reichardt, 2001a). The mechanisms by which distal NGF signaling is able to influence cell processes in the cell body, located a distance up to 1000x the diameter of the cell body, has been the topic of extensive research for many decades. Although several hypotheses were initially proposed for the retrograde transport of NGF signaling, including propagation of

waves of TrkA activity along the cell surface of the axon (Ginty and Segal, 2002, Senger and Campenot, 1997), the best supported model involves internalization of TrkA-NGF into a signaling endosome that is retrogradely transported to the cell body. There are numerous studies to support the transport of NGF and TrkA in an active complex, including co-localization, co-immunoprecipitation, and accumulation of labeled NGF (applied to targets in vivo) back in the cells body. Furthermore application of inhibitors to proximal axons did not impair the ability of distal NGF to elicit the accumulation of activated TrkA and downstream effectors in the cell body. While the precise mechanism of TrkA internalization is unknown, with evidence to support clathrin and caveolin -dependent and -independent endocytosis as well as micropinocytosis(Valdez et al., 2005, Ginty and Segal, 2002), it is clear that most TrkA internalization depends upon dynamin that mediates pinching of clathrin coated vesicles from the membrane (Ye et al., 2003, Zhang et al., 2000). Specifically, TrkA activation of PLCγ is necessary for calcineurin-mediated de-phosphorylation of dynamin-1 to promote TrkA internalization (Bodmer et al., 2011).

Once internalized, TrkA continues to associate with NGF leading to persistent signaling from endosomes. These TrkA signaling endosomes recruit effectors including small GTPases (Rab, Ras, Rap) and components of MAPK and PI3K signaling pathways to regulate trafficking of the receptor and local signaling. Interestingly, TrkA internalization appears to be required for both local NGF mediated axon growth as well as retrograde survival signaling (Bodmer et al., 2011). This may be due to sustained signaling which can occur from endosomes that are protected from silencing by membrane associated phosphatases or lysosomal degradation (Wu et al., 2001, Bodmer et al., 2011, York et al., 1998, York et al., 2000). Recent data has found that TrkA endocytosis is

required to activate the actin remodelers Rac1 and cofilin to "carve a path" through dense axonal actin to enable retrograde transport (Harrington et al., 2011). Although β-cells are not as highly polarized as neurons, we were curious as to whether TrkA endocytosis in β-cells is a conserved and necessary process through which neurotrophin signaling mediates glucose induced actin remodeling. We used a combination of surface biotinylation and antibody feeding techniques that have been well established in neurons to test whether or not TrkA internalization occurs in β-cells. We find that TrkA is endocytosed in β-cells and this process is required for GSIS and actin remodeling.

RESULTS

TrkA undergoes endocytosis in β-cells

In neurons, endosomal signaling from intracellular TrkA receptors is a key determinant of actin remodeling (Harrington et al., 2011). NGF promotes endocytosis of its TrkA receptors in nerve endings into signaling endosomes that are retrogradely transported to neuronal cell bodies to activate trophic signaling (Ascano et al., 2012). Endosomal TrkA signaling overcomes a dense peripheral actin network in axons to "carve a path" for retrogradely moving vesicles (Harrington et al., 2011). These findings raise the possibility of an analogous mechanism in β-cells where internalized TrkA receptors and neurotrophin signaling endosomes might mediate changes in F-actin necessary for insulin granule mobilization.

To address this possibility, we first asked whether TrkA receptors undergo ligand-dependent endocytosis in β-cells. We probed TrkA endocytosis using a cell surface biotinylation assay in the MIN6 mouse insulinoma cell line that exhibits many characteristics of primary β-cells including GSIS, and have been widely used for biochemical analyses (Ishihara et al., 1993). NGF stimulation for 30 minutes markedly increased TrkA endocytosis compared to un-stimulated cells **(Fig. 5-1A,B)**. Furthermore, we visualized the trafficking of surface TrkA receptors in primary β-cells by using a well-established antibody-feeding assay (Ascano et al., 2009). Primary β-cells were infected with an adenoviral vector expressing FLAG-tagged chimeric receptors that have the extracellular domain of TrkB and the transmembrane and intracellular domains of TrkA (FLAG-TrkB:A). The chimeric Trk receptors respond to the TrkB ligand, Brain-Derived

Neurotrophic Factor (BDNF), but retain the signaling properties of TrkA (Ascano et al., 2009). Live-cell immunocytochemistry with FLAG antibodies revealed predominantly surface localization of the receptors in the absence of ligand **(Fig. 5-1C)**. However, in BDNF-stimulated β-cells, Trk receptors accumulated in intracellular punctae that co-localized with Early Endosome Antigen 1 (EEA1), an early endosome marker **(Fig. 5-1D, E)**. Thus, ligand-mediated endocytosis of Trk receptors is a conserved mechanism that occurs in both pancreatic β-cells and neurons.

Mechanisms mediating TrkA internalization in β-cells

Previous work has shown that TrkA activation of PLCy1 is necessary for calcineurin mediated TrkA endocytosis (Bodmer et al., 2011). When either PLCy1 or calcineurin activity is blocked, NGF dependent TrkA internalization is significantly diminished in neurons. Interestingly we find that inhibition of PLCy1, but not calcineurin impairs internalization of biotinylated TrkA receptors in MIN6 cells **(Fig. 5-1F,G)**. In neurons, activation of calcineurin is required to interact with and de-phosphorylate dynamin-1ab to induce TrkA internalization. It is possible that this discrepancy in calcineurin dependence for TrkA internalization may be due to differences in expression of dynamin isoforms between β-cells and neurons (Raimondi, 2011, Lu et al., 2008).

Phosphorylation of TrkA receptors at a specific tyrosine residue, Y794, is necessary for PLCγ recruitment (Stephens et al., 1994). To assess the requirement of Y794 phosphorylation for TrkA endocytosis in β-cells, we performed antibody-feeding assays in MIN6 cells transfected with mutant FLAG-TrkAY794F or control FLAG-TrkA receptors. Upon NGF treatment, FLAG-TrkA receptors were found in intracellular punctae, whereas

mutant FLAG-TrkAY794F receptors remained largely at the cell surface **(Fig. 5-1H,I,J, and L)**. Furthermore, FLAG-TrkAY499F receptors, mutated at a site necessary for recruitment of the adaptor protein, Shc, and coupling to downstream MAP kinase and phosphatidylinositol 3-kinase (PI-3K) effector pathways, were internalized normally in response to ligand **(Fig. 5-1K and L)**. Thus, TrkA phosphorylation at Y794 and activation of PLCγ are selectively required for endocytosis in β-cells, as in neurons.

Endocytosed TrkA receptors mediate actin reorganization and insulin secretion

To investigate the effects of endocytosis-deficient Trk receptors on actin remodeling and insulin secretion in primary β-cells and islets, we generated adenoviral vectors expressing mutant FLAG-TrkAY794F or control FLAG-TrkA receptors, that were also doxycycline-inducible to precisely control expression. FLAG-tagged receptors were expressed in a doxycycline-dependent manner and also appeared normally on the cell surface in infected MIN6 cells **(Fig. 5-2A)**. To assess the effects of ectopic TrkA receptors on glucose-induced actin remodeling, isolated β-cells from TrkAF592A mice were infected with FLAG-TrkA adenoviruses, treated with doxycycline (100 ng/ml) for 24-30 hr, and then treated with 1NMPP1 for 20 minutes to silence endogenous TrkA activity. The FLAG-TrkA receptors are impervious to 1NMPP1 since they do not harbor the F592A mutation. β-cells were then exposed to low (2.8 mM) or high (16.7 mM) glucose, and F-actin visualized with phalloidin labeling. We found that ectopic expression of control FLAG-TrkA receptors promoted glucose-dependent actin reorganization in 1NMPP1-treated cells **(Fig. 5-1M, N and Q)**. In contrast, mutant FLAG-TrkAY794F receptors were unable to rescue the 1NMPP1-mediated impairment in actin reorganization **(Fig. 5-1O-Q)**.

In insulin secretion assays, control FLAG-TrkA receptors elicited robust insulin secretion in response to glucose, despite the presence of 1NMPP1 **(Fig. 5-1R)**. However, glucose-stimulated insulin secretion was suppressed in islets expressing mutant FLAG-TrkAY794F receptors **(Fig. 5-1R)**. Furthermore, PLCγ inhibition also decreased GSIS in islets, similar to the effects of the non-internalizable TrkA receptors **(Fig. 5-2B)**. These results suggest that TrkA endocytosis, via PLCγ activity, is required for glucose-stimulated actin remodeling and insulin secretion.

Mechanisms mediating TrkA internalization in β-cells

Previous work has shown that TrkA activation of PLCy1 is necessary for calcineurin mediated TrkA endocytosis (Bodmer et al., 2011). When either PLCy1 or calcineurin activity is blocked, NGF dependent TrkA internalization is significantly diminished in neurons. Interestingly we find that inhibition of PLCy1, but not calcineurin impairs internalization of biotinylated TrkA receptors in MIN6 cells **(Fig 5-1F,G)**. In neurons, activation of calcineurin is required to interact with and de-phosphorylate dynamin-1ab to induce TrkA internalization. It is possible that this discrepancy in calcineurin dependence for TrkA internalization may be due to differences in expression of dynamin isoforms between β-cells and neurons (Raimondi, 2011, Lu et al., 2008).

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mutant FLAG-TrkAY794F receptors remained largely at the cell surface **(Fig. 5-1H,I,J, and L)**. Furthermore, FLAG-TrkAY499F receptors, mutated at a site necessary for recruitment of the adaptor protein, Shc, and coupling to downstream MAP kinase and phosphatidylinositol 3-kinase (PI-3K) effector pathways, were internalized normally in response to ligand **(Fig. 5-1K and L)**. Thus, TrkA phosphorylation at Y794 and activation of PLCγ are selectively required for endocytosis in β-cells, as in neurons.

Investigating the dynamics of NGF signaling pathways in β-cells

In order to better understand how TrkA influences insulin secretion in β-cells, we analyzed the kinetic responses of canonical neurotrophin signaling pathways in MIN6 cells treated with NGF. We found that NGF is capable of activating PI3K, MAPK, and PLCγ signaling pathways in MIN6 cells **(Fig. 5-3A-F)**. While NGF elicited early and robust activation of ERK and PLCγ, p-AKT only showed significant phosphorylation after 1 hour of NGF stimulation. Interestingly CREB was also acutely activated within 10 minutes of NGF stimulation, which may contribute to the previous reports of pro-survival effects of neurotrophin signaling in β-cells. As activation of CREB is also known to stimulate MafA transcription to promote insulin production, future studies investigating the transcriptional effects of NGF on β-cells will be informative. In addition to acutely activating signaling effectors, TrkA in known to elicit prolonged signaling through the formation of signaling endosomes. One excellent example of this unique feature of TrkA signaling is the ability of TrkA, but not EGFR to activate the small GTPase Rap 1 in PC12 cells leading to continued MAP signaling up to an hour after stimulation (Wu et al., 2001). Given our findings that TrkA is internalized in β-cells, we tested that whether NGF could similarly

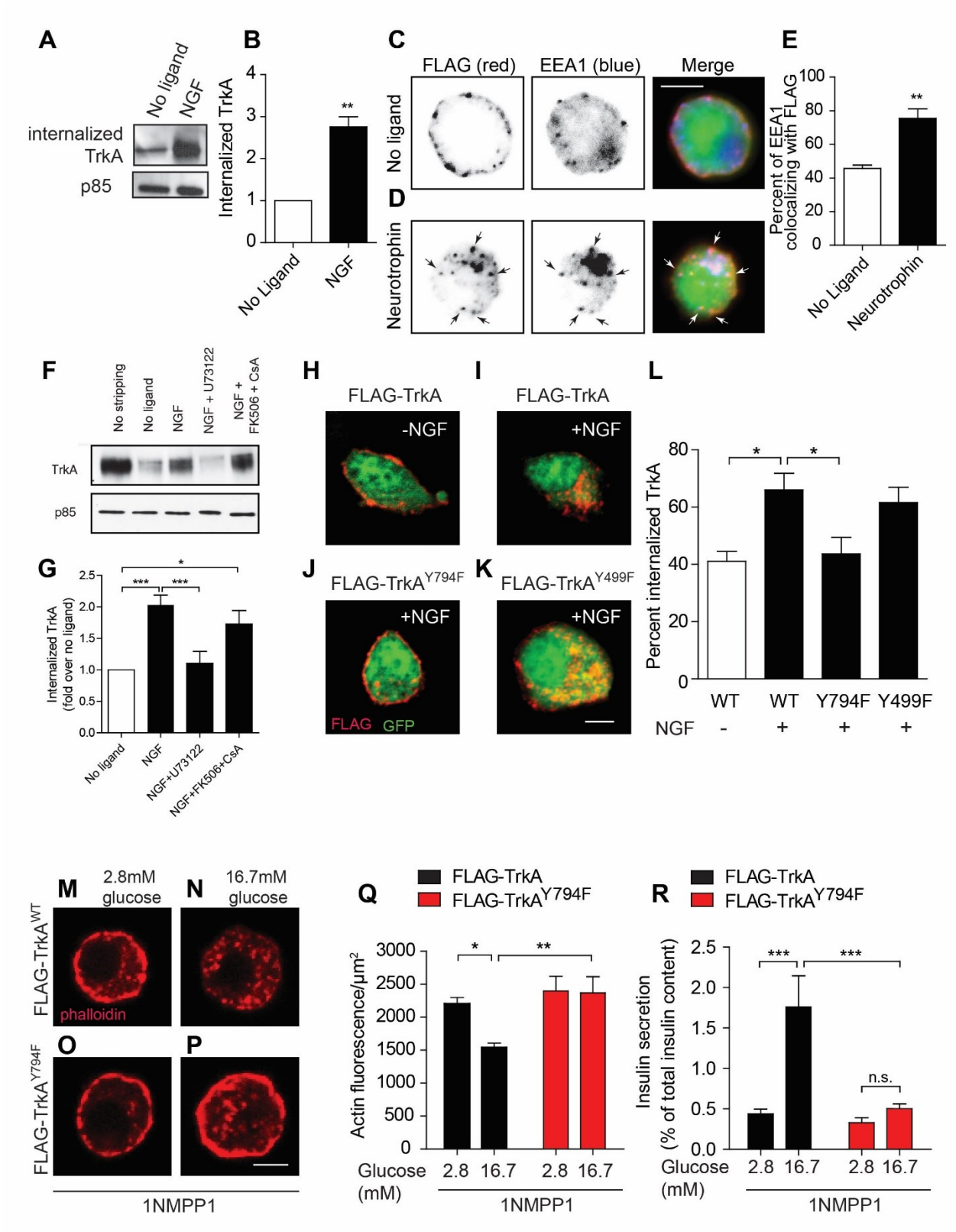
activate Rap1 in MIN6 cells. We found that indeed NGF activates Rap1 after one hour of treatment in MIN6 cells **(Fig. 5-4A,B)**. This result differs somewhat from findings in PC12 cells, however, in that activation is delayed and does not appear until 1 hour after stimulation, whereas NGF activates Rap1 robustly by 30 minutes in PC12 cells, perhaps indicating some differences in neurotrophin signaling kinetics between neurons and β-cells.

Figure 5-1: Glucose-induced actin changes and insulin secretion require TrkA endocytosis

(A) NGF (100 ng/ml, 30 minutes) promotes internalization of TrkA receptors in MIN6 cells. Membrane proteins were subjected to cell-surface biotinylation. Internalized TrkA receptors were detected by surface stripping of biotin, neutravidin precipitation, and TrkA immunoblotting. Supernatants were probed for p85 for normalization of protein amounts. **(B)** Densitometric quantification of internalized TrkA. Results are means ± SEM from 4 experiments, **p<0.01, *t*-test **(C,D)** Trk receptors undergo ligand-dependent internalization in primary β-cells, assessed by a live cell antibody feeding assay. β-cells were infected with an adenovirus expressing FLAG-TrkB:A chimeric receptors. Infected cells were identified by co-expression of GFP, which is cytoplasmic. Cells were labeled with FLAG antibodies under non-permeabilizing conditions at 4°C for 30 minutes, followed by stimulation with BDNF (100 ng/ml) for 10 minutes. Ligand stimulation enhanced the intracellular accumulation of Trk receptors, monitored by FLAG immunoreactivity (in red). Internalized Trk receptors accumulated in early endosomes marked by EEA1 (in blue). Scale bar, 5 μm. **(E)** Ligand-mediated internalization of chimeric receptors into early endosomes was determined by assessing the co-localization of EEA1 immunofluorescence with that of FLAG. At least 10-20 cells were analyzed per condition per experiment. Results are means ± SEM from 3 independent experiments. **p<0.01, *t*-test. **(F)** TrkA endocytosis requires PLCγ activity but not calcineurin in β-cells. A cell surface biotinylation assay was used to follow NGF-dependent internalization of TrkA receptors in MIN6 cells in the presence or absence of the PLCγ inhibitor, U73122 or inhibitors of calcineurin, FK506 and cyclosporin A. Total surface TrkA levels available for

biotinylation are shown in the “no stripping” lane. Supernatants were probed for p85 for normalization. **(G)** Densitometric quantification of internalized TrkA after treatments as described in **(F)**. Results are means ± SEM from 7 independent experiments. ***p<0.001, one way ANOVA followed by Tukey's post-test. **(H-K)** TrkA phosphorylation at Y794, the PLCγ docking site, is necessary for TrkA receptor endocytosis. Antibody feeding in MIN6 cells expressing FLAG-TrkA receptors shows that NGF-dependent receptor endocytosis is impaired by mutation of TrkA receptors at the PLCγ interaction site (Y794), but not Shc (Y499) binding site. Cells were labeled with FLAG antibodies under non-permeabilizing conditions at 4°C for 30 min, followed by stimulation with NGF (100 ng/ml) for 30 minutes. FLAG immunoreactivity is shown in red, and GFP, that was co-transfected, in green. Scale bar, 5 μm. **(L)** Internal accumulation of FLAG-TrkA receptors under the various conditions was determined by assessing the proportion of co-localization of FLAG immunofluorescence with that of GFP, which is co-expressed and is cytoplasmic. At least 10-20 cells were analyzed per condition per experiment. Results are the mean ± SEM from 5 independent experiments. *p<0.05, one way ANOVA followed by Tukey's post-test. **(M-Q)** TrkA receptor endocytosis is required for glucose-induced actin changes in primary β-cells. Expression of wild-type FLAG-TrkA receptors corrects 1NMPP1-mediated impairment of glucose-induced F-actin disassembly in isolated *TrkA*^{F592A} β-cells. However, mutant FLAG-TrkA^{Y794F} receptors that are unable to undergo internalization, fail to rescue defects in glucose-induced actin reorganization in 1NMPP1-treated cells. Scale bar, 5 μm. Quantification shows average fluorescence intensity for F-actin labeled with phalloidin. Values are mean ± SEM from n=6 independent experiments, 20-25 cells were analyzed per condition per experiment. *p<0.05, **p<0.01, two-way ANOVA with

Bonferroni post-test. **(R)** TrkA receptor endocytosis is required for insulin secretion in islets. Expression of wild-type FLAG-TrkA, but not FLAG-TrkA^{Y794F} receptors, rescues the 1NMPP1-mediated impairment of glucose-stimulated insulin secretion in *TrkA*^{F592A} islets. Values are mean ± SEM from n=5 independent experiments, ***p<0.001, n.s. not significant, two-way ANOVA with Bonferroni post-test.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NGF
internalized TrkA
p85
B
Internalized TrkA
No Ligand
NGF
C
FLAG (red)
EEA1 (blue)
Merge
No ligand
D
Neurotrophin
E
Percent of EEA1 colocalizing with FLAG
No Ligand
Neurotrophin
F
No stripping
No ligand
NGF
NGF + U73122
NGF + FK506 + CsA
TrkA
p85
G
Internalized TrkA (fold over no ligand)
No ligand
NGF
NGF+U73122
NGF+FK506+CsA
H
FLAG-TrkA
-NGF
I
FLAG-TrkA
+NGF
J
FLAG-TrkA^{Y794F}
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FLAG GFP
K
FLAG-TrkA^{Y499F}
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L
Percent internalized TrkA
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O
P
FLAG-TrkA^{Y794F}
1NMPP1
Q
FLAG-TrkA
FLAG-TrkA^{Y794F}
Actin fluorescence/μm^2
Glucose (mM) 2.8 16.7 2.8 16.7
1NMPP1
R
FLAG-TrkA
FLAG-TrkA^{Y794F}
Insulin secretion (% of total insulin content)
n.s.
Glucose (mM) 2.8 16.7 2.8 16.7
1NMPP1

Figure 5-2: Adenoviral expression of FLAG-TrkA receptors, and role of PLCγ activity in insulin secretion

(A) Doxycycline-induced expression of ectopic TrkA receptors in MIN6 cells infected with adenoviruses carrying FLAG-TrkA^{Y794F} or control FLAG-TrkA receptors. FLAG-tagged TrkA receptors appear on the cell surface as determined by a surface biotinylation assay. The FLAG immunoblot was stripped and reprobed for β-actin for normalization. **(B)** Glucose-stimulated insulin secretion is impaired in islets treated with a PLCγ inhibitor, U73122, compared to islets treated with an inactive analog, U73343. Values are means ± SEM from n=8 independent experiments, *p<0.05, ***p<0.001, n.s. not significant, two-way ANOVA with Bonferroni post-test.

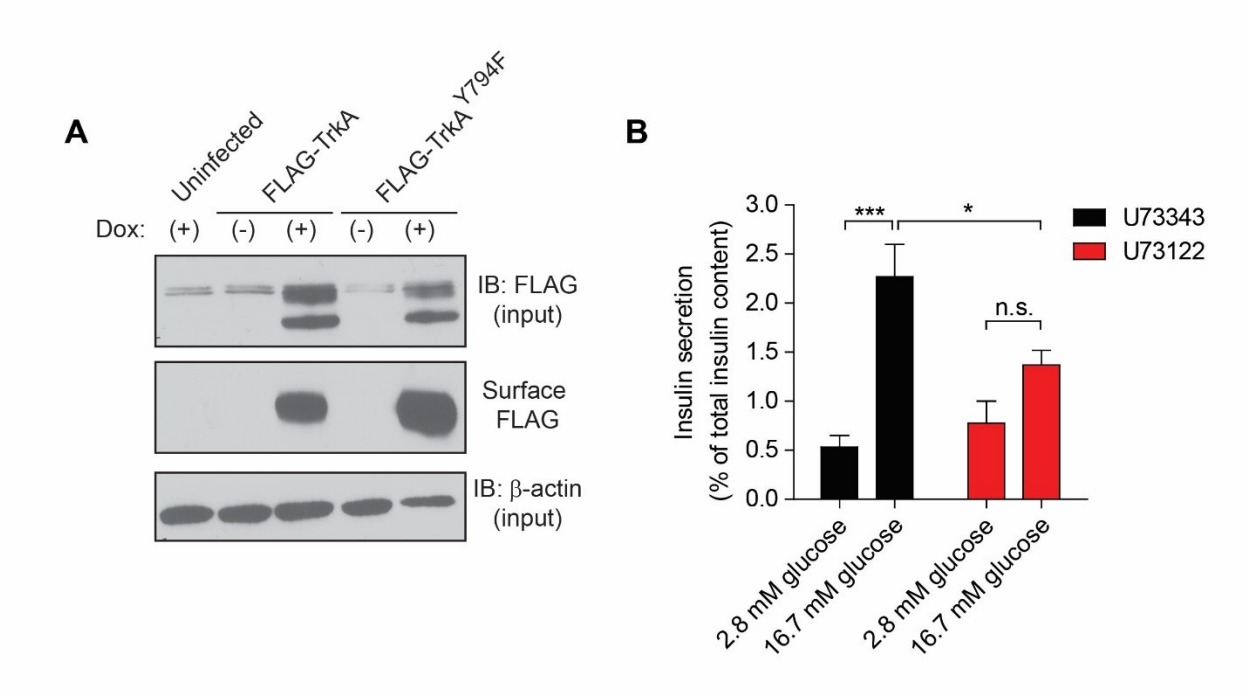
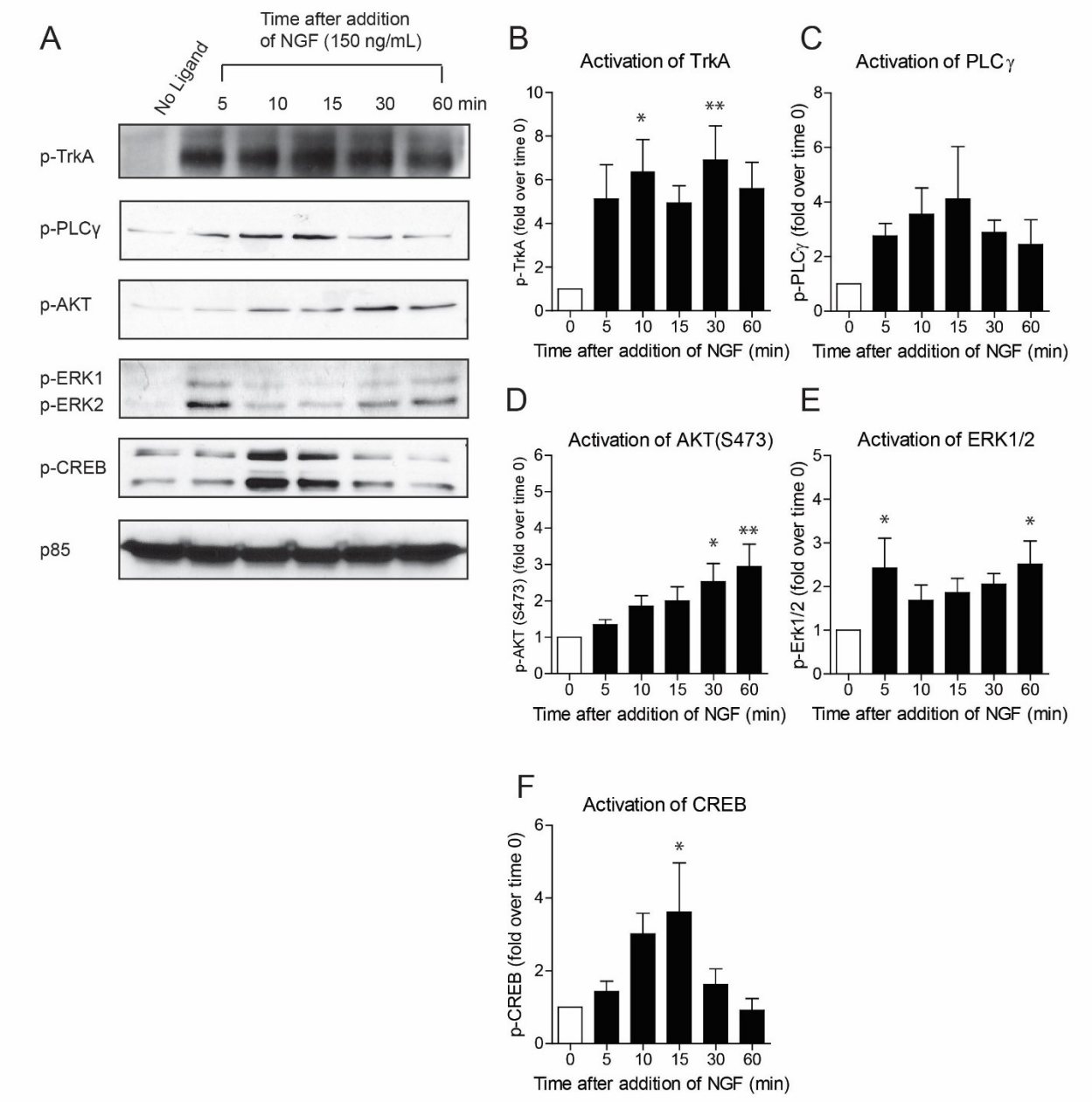


Figure 5-3: NGF activates TrkA and stimulates downstream signaling in MIN6 cells. **(A)** Equal numbers of MIN6 cells (passage 18-27) were incubated overnight in low serum and anti-NGF and the next day treated with either no ligand (time 0) or NGF (100 ng/mL) for 5, 10, 15, 30, or 60 minutes. Representative western blots are shown for p-TrkA, p-PLCγ, p-AKT (S473), p-ERK1/2, and p-CREB (S133). **(B)** Quantifications of NGF stimulation are normalized to control (0 minutes). TrkA shows significant activation after 10 and 30 minutes, 6.39±1.46 fold over 0 minutes, and 6.91±1.56 respectively. ERK1/2 is significantly activated after 5 and 60 minutes, 2.42±.68 and 2.56±0.53 respectively. AKT is activated 30 and 60 minutes after stimulation with NGF, 2.53±.50 and 2.94±0.62 respectively, and levels of p-CREB are significantly elevated after 15 minutes, 3.62±1.35 fold over time 0. Error bars represent means ± SEM, one-way ANOVA with Dunnett's multiple comparison test (compare with time 0) *$p<0.05$, **$p<0.01$ $n>5$ for all conditions.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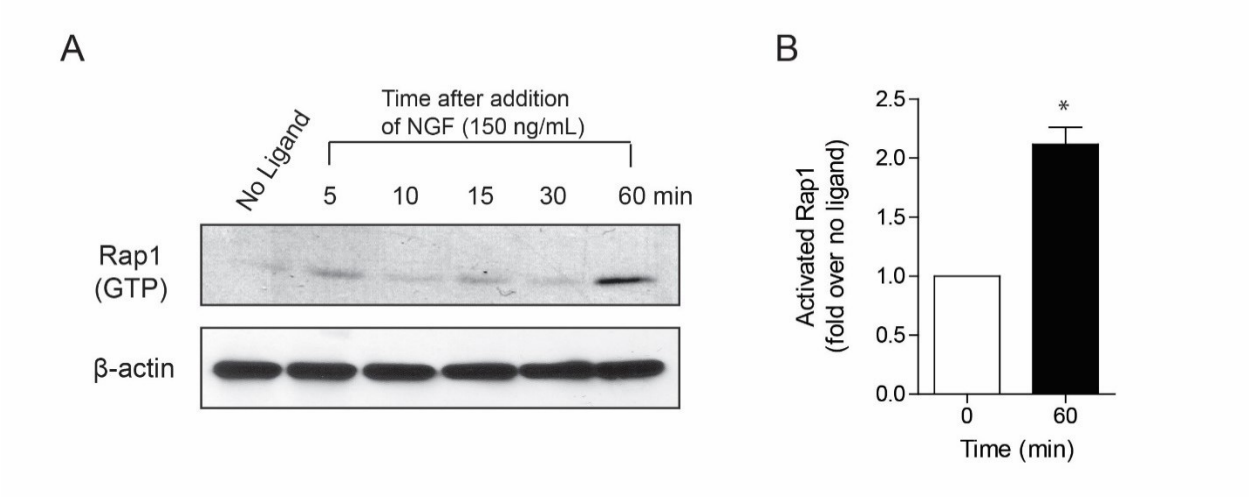
10
15
30
60 min
p-TrkA
p-PLCγ
p-AKT
p-ERK1
p-ERK2
p-CREB
p85
B
Activation of TrkA
p-TrkA (fold over time 0)
Time after addition of NGF (min)
C
Activation of PLCγ
p-PLCγ (fold over time 0)
Time after addition of NGF (min)
D
Activation of AKT(S473)
p-AKT (S473) (fold over time 0)
Time after addition of NGF (min)
E
Activation of ERK1/2
p-Erk1/2 (fold over time 0)
Time after addition of NGF (min)
F
Activation of CREB
p-CREB (fold over time 0)
Time after addition of NGF (min)

Figure 5-4: NGF activates Rap1 MIN6 cells. (A) Equal numbers of MIN6 cells (passage 18-27) were incubated overnight in low serum and anti-NGF and the next day treated with either no ligand (time 0) or NGF (100 ng/mL) for 5, 10, 15, 30, or 60 minutes. Representative western blots are shown for Rap1-GTP. **(B)** Quantifications of NGF stimulation are normalized to control (0 minutes). TrkA shows significant activation after 60 minutes, 2.11±.0.14. Error bars represent means ± SEM, one-sample t-test *p<0.05, n = 3.



DISCUSSION

In neurons, TrkA internalization in axon terminals, and subsequent endosomal signaling, is obligatory for long-distance communication between distal axons, the site of action of target-derived NGF, and neuronal cell bodies, as recently reviewed in (Cosker and Segal, 2014). In β-cells, TrkA endocytosis may allow access to intracellular signaling effectors including actin modulatory proteins, prolong receptor activation, or allow localized responses at specific sub-cellular domains (Sorkin and von Zastrow, 2009). Internalized TrkA endosomes could function to carry actin remodeling signals to the cell interior to promote the movement of insulin granules to the surface during the second phase of insulin secretion.

Interestingly, a recent study showed that β-cell specific deletion of dynamin 2 results in a striking increase in F-actin density and impaired insulin secretion and glucose tolerance (Fan et al., 2015). Given our similar observations with TrkA inactivation, and the apparent calcineurin-dynamin 1 independence of TrkA internalization in β-cells, this raises the possibility that TrkA receptors may be a cargo for dynamin 2-mediated endocytosis in β-cells. Previous work has established that β-cells express both dynamin 1 and 2 (Lu et al., 2008), however, a more extensive analysis of the specific isoforms of each dynamin variant will be important in determining the exact regulation of TrkA internalization in β-cells.

We found that, similar to NGF stimulation in neurons, TrkA signaling activates Rac1 however, TrkA may also associate with other actin remodelers. MAPK signaling originating from TrkA endosomes could also contribute to actin remodeling via an established role in promoting phosphorylation and activation of MLCK and MyoIIA

(Arous et al., 2013). While we have highlighted an acute role for TrkA signaling in GSIS, long-term signaling may have functions in transcriptional responses, proliferation, or survival of β-cells. Previous studies using neutralizing NGF antibodies, or pharmacological inhibition attenuated survival of rat β-cells in culture (Navarro-Tableros et al., 2004), while infusion of NGF increased islet mass and viability in transplanted islets in diabetic rats (Reimer et al., 2003)

This work focuses on the effect of TrkA signaling on actin remodeling via the formation of a signaling endosome however, we cannot rule out the possibility that TrkA signaling in β-cells mediates actin dynamics through PLCγ, independently of endocytosis. PLC signaling is known to promote exocytosis in chromaffin cells through the production of DAG that interacts with the priming factor Munc13-1, which is also important for sustained insulin secretion in β-cells (Bauer et al., 2007). PLC-mediated decreases in PIP_2 have been implicated in actin remodeling (Yamaga et al., 2015) and growth factor stimulation of PLC has been shown to activate cofilin in migrating carcinoma cells (van Rheenen et al., 2007). In this way, local and rapid TrkA activation of PLCγ may also directly contribute to the activation of gelsolin or insulin granule docking machinery proximal to the plasma membrane.

Although we have found an essential role for the TrkA-PLCγ signaling cascade in mediating actin remodeling, it is possible that TrkA-mediated activation of other pathways (namely MAPK, which has also been shown to be activated by phosphorylation on TrkAY794 (Stephens et al., 1994)) could augment signaling leading to actin remodeling in response to glucose. Recently the Src kinase, YES was shown to be activated at the plasma membrane in response to glucose (Yoder et al., 2014). Although there is no evidence yet

for TrkA interaction with this specific Src kinase, it has been shown that Src kinases can augment TrkA signaling in response to NGF (Tsuruda et al., 2004, Shi et al., 2010). Additionally, TrkA has been shown to interact with ephrinA5 in PC12 cells (Marler et al., 2008), leading to the interesting possibility that TrkA could be involved in regulated EphA5/ephrinA5 signaling in β-cells which is known to be important for actin remodeling and insulin secretion (Konstantinova et al., 2007). Perhaps other glucose stimulated signaling effectors converge on TrkA signaling to help mediate the effects of NGF on TrkA in response to glucose.

METHODS

Mice and cell lines

All procedures relating to animal care and treatment conformed to Johns Hopkins University Animal Care and Use Committee (ACUC) and NIH guidelines. Mice were housed in a standard 12:12 light-dark cycle. Mice were maintained on a *C57BL/6* background, or mixed *C57BL/6* and *129P*, or *C57BL/6* and *FVB* backgrounds. Both sexes were used for analyses at 1-2 months of age, unless stated otherwise in the figure legends. $TrkA^{F592A}$ ($TrkA^{f/f}$) mice were obtained from the Jackson Laboratory (Stock No: 022362).

The MIN6 cell line was a generous gift from Dr. Jun-ichi Miyazaki (Osaka University) and Dr. Donald Steiner (University of Chicago). Cells between passage 12-30 were maintained in DMEM media plus 10% FBS, 5 U/L penicillin/5µg/L streptomycin, 200mM L-glutamine, 100mM Sodium pyruvate, and 2µM β-mercaptoethanol.

Antibodies

Primary antibodies for immunofluorescence include guinea pig anti-insulin (1:300, abcam ab7842), mouse anti-EEA1 (1:500, BD Biosciences 610457). Rabbit anti-FLAG (1:100, Sigma Aldrich F7425) and mouse M2 anti-FLAG (1:100, Sigma Aldrich F3165) were used for live-cell antibody feeding assays. Fluorescent secondary antibodies were Alexafluor-350, -488,-546, or -647-conjugated and used at 1:200 (Invitrogen)

Primary antibodies for western blots include rabbit anti-phospho-AKT (S473) (1:1000 Cell Signaling 9271S), mouse anti-phospho-ERK1/2 (Thr202/Tyr204) (1:1000 Cell Signaling 9106), rabbit anti-phospho-CREB (S133) (1:1000 Cell Signaling 9198), rabbit anti-PLCγ1 (1:1000 Cell Signaling 2822), rabbit anti-TrkA (1:1000, Millipore 06-574), rabbit anti-Rap1 (Millipore 07-916), rabbit anti-p85 (1:3000, Upstate Biotechnology 06-195), and rabbit anti-β-actin (1:1000, Cell Signaling, 4970)

MIN6 cell NGF stimulation time course

MIN6 cells were seeded at 0.8x106 cells/well in 6-well dishes and allowed to recover overnight. Following recovery, cells were starved in media containing 0.5% FBS. The next morning, cells were pre-incubated in DMEM without serum for 1 hour before stimulation with NGF (100ng/mL) for the indicated periods of time. The zero NGF control plate was maintained in DMEM without NGF for one hour. After incubation, cells were washed briefly with PBS and lysed with 500 µl RIPA buffer (50mM Tris-HCl, 150mM NaCl, ImM EDTA, 1% NP-40, 0.25% deoxycholate). For detection of p-TrkA and p-PLCγ, lysates were immunoprecipitated with 1:100 mouse anti-phospho-Tyrosine (clone PY20) and 1:10 protein A beads (Santa-Cruz) rocking at 4ºC for 2 hours. Supernatants were collected for analysis of p-AKT, p-ERK1/2, p-CREB, and p85 for normalization of protein amounts. Beads were washed 3 times with RIPA before resuspension in 1x Laemmli buffer. Samples were boiled for 5 min prior to loading and SDS-PAGE analysis. Following transfer, membranes were probed with primary antibodies and then HRP-conjugated secondary antibodies.

For detection of active Rap1-GTP, cell lysates were immunoprecipitated using a Rap1 activation assay kit (Millipore 17-321) according to the provider's instructions. In brief, cells were lysed Rap1 activation lysis buffer, and lysates were incubated with Ral GDS-RBD agarose beads for 1 hour at 4ºC with rocking. Supernatants were collected for protein normalization and beads were washed 3 times with lysis buffer and resuspended in 1X Laemmli buffer. Supernatant and precipitated samples were subjected to SDS-PAGE and following transfer, membranes were probed for Rap1, and β-actin for protein normalization.

All immunoblots were visualized with ECL Plus Detection Reagent (Thermo Scientific, 32132) and scanned with a Typhoon 9410 Variable Mode Imager (GE Healthcare). Densitometric analysis of bands was performed using ImageJ software.

Plasmids and adenoviral vectors

FLAG-TrkA^{Y794F} and FLAG-TrkA^{Y499F} constructs were generated by point mutations in a parent FLAG-TrkA vector (Bodmer et al., 2011), using QuikChange II XL site-directed mutagenesis kit (Agilent, #200521). Mutant plasmids were verified by DNA sequencing. Recombinant adenoviruses expressing FLAG-TrkA or FLAG-TrkA^{Y794F} were generated by sub-cloning FLAG-TrkA or FLAG-TrkA^{Y794F} from PCDNA3.1 into pAdenoX-Tet3G using the Adeno-X^{TM} Adenoviral System 3 kit (Clontech). Recombinant adenoviral backbones were packaged into infectious adenoviral particles by transfection into HEK 293 cells (ATCC) using Lipofectamine (Invitrogen). Generation of adenovirus expressing

chimeric FLAG-TrkB:TrkA receptors have been described previously (Ascano et al., 2009). High-titer viral stocks were purified using a CsCl gradient.

Islet isolations

Islets were isolated as previously described (Wollheim et al., 1990). Briefly, pancreata collected from 1-2 month old mice were distended using collagenase (Collagenase P, 0.375 mg/ml, Roche) dissolved in Hank's Balanced Salt Solution (HBSS, Mediatech), and digested at 37°C. Digested pancreata were washed with HBSS, and subjected to discontinuous density gradient centrifugation using varying histopaque densities (Sigma-Aldrich). The islet layer was collected, washed with HBSS, and islets handpicked under an inverted microscope for subsequent analyses.

TrkA receptor internalization assays

Cell surface biotinylation assays were performed in MIN6 cells as previously described (Kuruvilla et al., 2004). Briefly, MIN6 cells were biotinylated at 4ºC with a reversible membrane-impermeable form of biotin (EZ-Link™ NHS-SS-Biotin, 1.5mg/ml in PBS, ThermoFisher Scientific, Cat# 21441) for 25 minutes. For PLCγ inhibition, MIN6 cells were pre-treated for 30 minutes with U73122 (10 μM, Sigma Aldrich) or cyclosporine A and FK506 (2μg/mL/0.2μg/mL). Cells were washed briefly with PBS containing 50mM glycine (Sigma) to remove remaining unconjugated biotin, and then moved to 37ºC in DMEM containing 10% FBS and 5 U/l penicillin/streptomycin ± NGF (100ng/ml) for 30 minutes to promote internalization. Cells were returned to 4ºC, the remaining biotinylated surface receptors were stripped of their biotin tag with 50mM glutathione (Sigma),

followed by two washes with 50mM iodoacetamide (Sigma) to quench excess glutathione. Cells were lysed with 500µl of RIPA buffer, and supernatants subjected to precipitation with 40µl-immobilized neutravidin agarose beads (ThermoFisher Scientific) and immunoblotted for TrkA.

For expression analysis of the FLAG-TrkA control and FLAG-TrkAY794F receptors, MIN6 cells were infected with adeno-expressing FLAG-TrkA or FLAG-TrkAY794F viruses and induced with or without doxycycline (100 ng/ml) in RPMI 1640 media containing 5% FBS and 5 U/l penicillin/streptomycin for 24 hours. Cell surface biotinylation was performed as described above and cells were immediately washed with cold PBS and glycine, lysed with RIPA, and subjected to precipitation with neutravidin agarose beads. Supernatant and precipitated samples were subject to SDS-PAGE and probed for TrkA.

Live cell antibody feeding assays were performed in dissociated β-cells and MIN6 cells as previously described (Ascano et al., 2009). βeta-cells were dissociated by gentle trituration in HBSS containing 4 mM EDTA, plated on poly-D lysine-coated coverslips, and transduced with an adenovirus expressing FLAG-TrkB:A chimeric receptors. Infected cells identified by GFP expression. Cells were rinsed briefly with PBS and incubated with rabbit anti-FLAG antibody (1:100) for 30 minutes at 4ºC, followed by a brief wash with cold PBS, and then stimulated with or without BDNF (100 ng/ml) in DMEM for 10 minutes at 37ºC. Cells were quickly washed with PBS, fixed in 4%PFA/PBS for 30 minutes at room temperature, and permeabilized in 0.1% Triton X-100/1% BSA/PBS for 30 minutes at

room temperature. Immunostaining for insulin and EEA1 were performed overnight at 4ºC. Cells were washed in PBS and fluorescently conjugated secondary antibodies were applied for 1 hour at room temperature, washed again in PBS, and mounted on slides with Fluoromount. Images representing 1 μm optical slices were acquired using a Carl Zeiss LSM 510 confocal equipped with Confocor 3 Fluorescence Correlation Spectroscopy module, and argon (458–488 nm) and helium/neon (543–633) lasers. The same confocal acquisition settings were applied to all images taken from a single experiment. Threshold settings for blue and red scans were determined, and internalization was quantified as the ratio of anti-FLAG immunofluorescence (red) that colocalized with EEA1 (blue) relative to the total anti-FLAG immunofluorescence. Weighted coefficients of co-localization between the anti-FLAG and EEA1 fluorescence were determined by ImageJ software. 10-20 cells were analyzed per condition per experiment. For antibody feeding assays in MIN6 cells, cells were first transfected with plasmids carrying FLAG-TrkA, FLAG-TrkA^{Y794F}, or FLAG-TrkA^{Y499F}. GFP was co-transfected in all cases. Cells were stimulated with NGF for 30 minutes or left untreated in media alone. Internalization was quantified as the ratio of anti-FLAG immunofluorescence (red) that co-localized with cytoplasm (GFP signal) relative to the total anti-FLAG immunofluorescence for 10-15 cells per experiment.

Phalloidin staining

Islets were isolated from adult 1-2 month old *TrkA^{F592A}* mice and dissociated into single cells by gentle trituration in HBSS containing 4 mM EDTA. Cells were resuspended in RPMI 1640 media containing 5% FBS and 5 U/l penicillin/streptomycin, and plated on glass coverslips coated with poly-D lysine. After 24 hours, islet cultures were treated with

1NMPP1 (20 μM) or DMSO for 20 minutes, followed by incubation in low (2.8 mM) or high (16.7 mM) glucose for another 30 minutes. Cells were fixed in 4% PFA and stained with Alexa546–conjugated phalloidin (Invitrogen). β-cells were identified by immunostaining for insulin. Images representing 1μm optical sections were acquired using a Zeiss LSM 510 confocal microscope. Average fluorescence intensities of Alexa-546 per μm^2 in individual β-cells were measured using ImageJ software (NIH). 20-25 cells were analyzed per condition per experiment.

To assess the effects of endocytosis-defective TrkA receptors on F-actin, isolated β-cells from *TrkA*^{F592A} mice were incubated with adenoviruses expressing either FLAG-TrkA^{Y794F} or control FLAG-TrkA receptors, and treated with doxycycline (100 ng/ml) in RPMI 1640 media containing 5% FBS and 5 U/l penicillin/streptomycin for 24 hours. β-cells were then incubated in 1NMPP1 (20μM) for 20 minutes to silence endogenous TrkA receptors, and then fixed and labeled with Alexa546–phalloidin as described above.

***In vitro* GSIS assays**

To assess the effects of endocytosis-defective TrkA receptors on GSIS, *TrkA*^{F592A} islets were incubated with high-titer adenoviruses expressing either FLAG-TrkA^{Y794F} or control FLAG-TrkA receptors, and treated with doxycycline (100 ng/ml, Sigma) in RPMI 1640 media containing 5% FBS and 5 U/l penicillin/streptomycin for 30 hours. Islets were washed in Krebs-Ringer HEPES buffer (KRHB) containing low (2.8 mM) glucose and allowed to stabilize for 1 hour. Islets were then pre-incubated with 1NMPP1 (20μM) for 20 minutes to silence endogenous TrkA592A receptors. Groups of 5-10 islets were then

handpicked into 24-well dishes and incubated in low (2.8 mM) or high glucose (16.7 mM) in KRHB buffer for another 30 minutes. Supernatant fractions were removed, the islets were lysed in acid ethanol, followed by insulin ELISA (Crystal Chem) to determine the insulin concentrations in both supernatant and islet fractions.

CHAPTER 6. GLUCOSE DEPENDENT REGULATION OF NGF SECRETION AND TRKA ACTIVITY, AND IMPLICATIONS FOR HUMAN ISLET FUNCTION

Portions of this chapter were previously submitted for publication at Developmental Cell: Jessica Houtz, Philip Borden, Alexis Ceasrine, and Rejji Kuruvilla (2016). *Neurotrophin signaling is required for glucose-induced insulin secretion.*

INTRODUCTION

Extrinsic regulators of insulin secretion

Although loss of β-cell function precedes a reduction in β-cell mass during the development of diabetes (Defronzo, 2009, Weir and Bonner-Weir, 2004), research has predominantly focused on mechanisms governing β-cell proliferation (Vetere et al., 2014, Wang et al., 2015, Stewart et al., 2015), and far less is known about extrinsic factors that influence GSIS. While glucose is the primary and most potent stimulator of insulin secretion, neurotransmitters (Woods and Porte, 1974, Ahrén et al., 2006) and incretins (Drucker, 2006) also have well-established roles as insulin secretagogues. They influence GSIS by generating second messengers that impinge on β-cell electrical activity and/or insulin exocytosis (Ahrén et al., 2006, Nakazaki et al., 2002, Drucker, 2006).

Some nutrient-derived factors, such as amino acids and fatty acids, are metabolized by β-cells and stimulate ATP production to augment insulin secretion (McClenaghan et al., 1996, Roduit et al., 2004, Prentki et al., 1992). Recently, many of these same nutrients have also been found to potentiate insulin secretion by acting directly on G-protein coupled receptors (GPCRs) on β-cells to increase intracellular calcium (Pi et al., 2012, Itoh et al., 2003, Briscoe et al., 2003). Perhaps the most well characterized insulin regulators are metabolic hormones known as incretins, namely glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide (GIP), which are secreted by L- and K-intestinal cells respectively. These peptide hormones act through GPCRs to increase intracellular cAMP in pancreatic β-cells resulting in the activation of PKA and Epac2 (Holz, 2004, Drucker, 2006) which modulate intracellular calcium (Kang et al., 2005), closure of K_{ATP} channels

(Nakazaki et al., 2002), and phosphorylation of granule fusion machinery including Snapin and synaptogamin-7(Wu et al., 2015, Song et al., 2011) The overall effect of incretins on these regulators of insulin secretion is to increase the pool of readily releasable granules to enhance secretion upon stimulation (Wan et al., 2004, Shibasaki et al., 2007). Beyond their insulinotropic effects, incretins also regulate transcription via activation of cyclic response element binding protein (CREB) which promotes β-cell survival (Kim et al., 2005, Kim et al., 2008) and proliferation (Buteau et al., 2003, Xu et al., 1999).

Given the potency of insulin signaling, it is not surprising that many insulin secretagogues are themselves precisely regulated by nutrient intake to protect against hypoglycemia and ensure that insulin secretion *in vivo* remains glucose-dependent at much lower glucose concentrations than are effective *in vitro* (Henquin et al., 2003). Vagal activity during the meal anticipatory "cephalic phase" of insulin secretion leads to increased secretion of gastrin, ghrelin, and GLP-1, as well as insulin to help prime the body for optimum nutrient absorption and maintenance of glucose homeostasis (Power and Schulkin, 2008). Following meal consumption, an even greater increase in circulating levels of many enzymes and metabolic hormones is observed (Begg and Woods, 2013). These signals come predominantly from the gastrointestinal tract and influence meal size by producing signals that act either indirectly through the vagal pathway, or directly by activating receptors in the brain (Begg and Woods, 2013). In addition to incretin hormones, decretins such as neuromedin U and galanin, help keep insulin secretion low during periods of nutrient deprivation (Hussain et al., 2016). Signaling from the liver and adipose tissue, via kisspeptin and leptin respectively, can also impair GSIS when there is excessive nutrient intake. Stress and inflammatory factors, such as IL-6 and TNF, are known to

influence both food intake and the production of incretins, which likely contribute to aberrant regulation of insulin secretion in diseased states (Ellingsgaard et al., 2011, Zietek and Rath, 2016).

Neurotrophins in metabolism and energy homeostasis

Neurotrophins have been best studied for their influence on neuronal development, however, they have also been found to play important roles in the regulation of feeding and metabolism. Perhaps the most extensively characterized neurotrophin in metabolic function is BDNF. Recent data has highlighted the importance of BDNF both within several nuclei of the hypothalamus that are responsible for regulating appetite and energy balance as well as in the periphery within glucagon secreting alpha cells. Loss of BDNF or TrkB in mice has been shown to result in hyperphagia, obesity, and increased anxiety (Rios et al., 2001, Kernie et al., 2000, Yang et al., 2016, Xu et al., 2003). Conversely, administration of BDNF reduces food intake and lowers blood glucose levels in mouse models of obesity and diabetes (Ono et al., 1997, Tonra et al., 1999). In clinical studies in humans, plasma levels of BDNF are found reduced in cases of atherosclerosis, metabolic syndrome, and type two diabetes (Manni et al., 2005, Boyuk et al., 2014, Civelek et al., 2013, Suwa et al., 2006, Chaldakov et al., 2004).

Both expression of BDNF and its high affinity receptor, TrkB are enriched in several regions of the hypothalamus in the adult brain, including the ventromedial (VMH), arcuate (ARC), paraventricular (PVN) nuclei, that are key regulators of energy homeostasis and feeding behavior (Yang et al., 2016, An et al., 2015, Xu et al., 2003). In the VMH, BDNF expression is regulated by the melanocortin-4 receptor activity in response to

nutritional state. Under fasting conditions and low nutrient availability, MC4R activity and BDNF expression are repressed, but in response to feeding or stimulation with a MC4R agonist, BDNF levels increase. Interestingly, the specific effect of BDNF on lowering blood glucose levels was shown to be independent of the reduction in food intake by eloquent paired-feeding experiments (Nakagawa et al., 2000, Yamanaka et al., 2006, Coppola and Tessarollo, 2004). Experiments to determine how BDNF might regulate glucose homeostasis revealed that BDNF had no influence on insulin levels. Rather, BDNF was found to decrease the concentration of circulating glucagon, thus inhibiting aberrant glucose mobilization (Yamanaka et al., 2006, Hanyu et al., 2003). TrkB immunoreactivity is present in alpha cells in the mantle of murine islets, but not in the core occupied by β-cells (Hanyu et al., 2003). Furthermore, administration of BDNF in isolated islets decreased glucagon secretion. In addition to directly modulating alpha-cell activity, BDNF signaling through TrkB in adipocytes may regulate transcriptional regulation of "browning" of white fat (Cao et al., 2011).

Although the effects of BDNF/TrkB signaling on regulation of energy homeostasis and feeding have been extensively studied, less is known about the involvement of other neurotrophins in metabolic control. Despite this paucity of information, studies have revealed potential metabolic roles for ciliary neurotrophic factor (CNTF) and NGF. Originally identified as a factor necessary for the survival of ciliary ganglion cells, CNTF was found to promote neurogenesis in POMC expressing neurons and inhibit the expression of orexigenic NPY in the hypothalamus (Kokoeva et al., 2005). In the periphery, there is evidence that CNTF can act on skeletal muscle, brown fat, and the liver to increase fatty acid oxidation, upregulate makers of brown fat, and improve insulin sensitivity. In

animal models CNTF was shown to improve symptoms associated with obesity and diabetes (Liu et al., 2007, Gloaguen et al., 1997). The clinical relevance of these findings was exemplified by the observed improvement in hyperglycemia, hyperinsulinemia, and hyperlipidemia in obese individuals treated with a human recombinant variant of CNTF (Axokine®) (Ettinger et al., 2003).

Dysregulation of NGF has also been observed in patients diagnosed with atherosclerosis, metabolic syndrome, and diabetes (Manni et al., 2005, Civelek et al., 2013, Bullo et al., 2007, Chaldakov et al., 2004, Anand et al., 1996). During the initial stages of metabolic syndrome, it has been hypothesized that the interplay of cytokines and neurotrophins leads to hyper-activation of the autonomic nervous system resulting in eventual hormonal imbalance and peripheral leptin and insulin insensitivity (Hristova and Aloe, 2006). Indeed, NGF has been documented to activate the pituitary-adrenocortical axis in response to stress resulting in increased circulating glucocorticoids (Otten et al., 1979, Taglialatela et al., 1991). Once metabolic syndrome is fully manifested, a decrease in plasma levels of neurotrophins is observed (Chaldakov et al., 2001, Chaldakov et al., 2004, Hristova and Aloe, 2006). Together these studies highlight the importance of neurotrophins as metabolic regulators of food intake and energy homeostasis.

RESULTS

Alterations in NGF levels have been noted in serum, nerves, and peripheral tissues in diabetic humans and animal models (Bullo et al., 2007) (Bullo et al., 2007, Kim et al., 2009, Meloni et al., 2010). Additionally, elevated glucose enhances NGF biosynthesis and secretion in cultured rat β-cells (Rosenbaum et al., 1998). These results, together with the observed expression patterns of NGF and TrkA in the pancreas, prompted us to ask if the NGF signaling pathway might be regulated by glucose *in vivo*. To address this question, we measured serum NGF levels in mice that were fasted overnight and subjected to a glucose challenge administered intra-peritoneally. We observed a significant elevation in circulating NGF levels within 15 minutes of the glucose challenge **(Fig. 6-1A)**, suggesting that NGF secretion is acutely regulated by glucose *in vivo*.

To determine if glucose influences TrkA activity, we assessed TrkA phosphorylation in isolated islets treated with high glucose (16.7mM). 15 minutes of exposure to elevated glucose stimulated a robust increase (11.69 ± 1.9-fold) in islet TrkA phosphorylation levels, compared to basal conditions (2.8 mM glucose) **(Fig. 6-2A,B)**. Together, these findings indicate that NGF secretion and signaling in pancreatic islets are regulated by glucose, and suggest an instructive role for the NGF pathway in facilitating GSIS.

Glucose dependent NGF-TrkA signaling in human islets

Basic research performed in rodent model systems has yielded significant insights into the mechanisms and key regulatory pathways involved in GSIS in humans, however, as with any model system, there are limits to the parallels that can be drawn between

processes occurring in one organism versus the other. Recent studies have begun to highlight structural, transcriptional, and functional differences between human and rodent islets (Jiao et al., 2014, Cabrera et al., 2006, Benner et al., 2014, MacDonald et al., 2011). These results demonstrate the importance of determining the relevance of findings in mouse models in a human system. The advent of organizations that improve the accessibility and distribution of human islets to investigators (such as the Integrated Islet Distribution Program-IIDP in the U.S.A.) (Kulkarni and Stewart, 2014, Kaddis et al., 2009), and techniques for generating β-cells from induced pluripotent stem cells (Hua et al., 2013) has made it more feasible to make the needed comparisons. We acquired human islets from the IIDP from 6 healthy individuals of both sexes between the ages of 33 and 59 and 3 and pre-diabetic individuals, and tested the effect of glucose on TrkA activity and GSIS. Similar to our findings in mouse islets, we see significant enhancement of TrkA phosphorylation in human islets in response to an acute elevation in glucose **(Fig. 6-3A,B)**. Although the level of stimulation of p-TrkA is not as great in human islets, human islets were maintained under culture conditions for several days as opposed to overnight culturing in mouse islets. It is possible that relevant cell types that produce NGF in human islets may be compromised by this prolonged period of culturing, thus affecting the level of stimulation in response to glucose. Interestingly, stimulation with high glucose in islets from pre-diabetic individuals had no effect on p-TrkA levels, implying that TrkA signaling is affected in a diseased state. Since pre-diabetic individuals are defined by having higher than normal blood glucose levels, but are not overtly diabetic, impaired neurotrophin function may be an early indicator of reduced β-cell function. Additionally, we found that NGF treatment augmented GSIS in human islets, but basal insulin secretion was unaffected

(Figure 6-1G). These results support a physiological role for NGF in potentiating GSIS, although NGF is insufficient by itself to trigger insulin secretion.

Figure 6-1: Glucose regulates NGF secretion from smooth muscle cells

(A) Circulating NGF levels are rapidly increased 15 minutes after a glucose injection (2 g/kg body weight, i.p.) in mice. Mice were fasted overnight prior to the glucose challenge. NGF levels were measured by ELISA, from tail vein blood drawn before and 15 minutes after glucose administration. Values are the mean ± SEM from n=14 mice, *p<0.05, paired two-tailed *t*-test. **(B)** Cultured aortic smooth muscle (P3) secretes NGF in response to elevated glucose (15 minutes). Values are the mean ± SEM, n= 2 *p<0.05, one sample *t*-test.

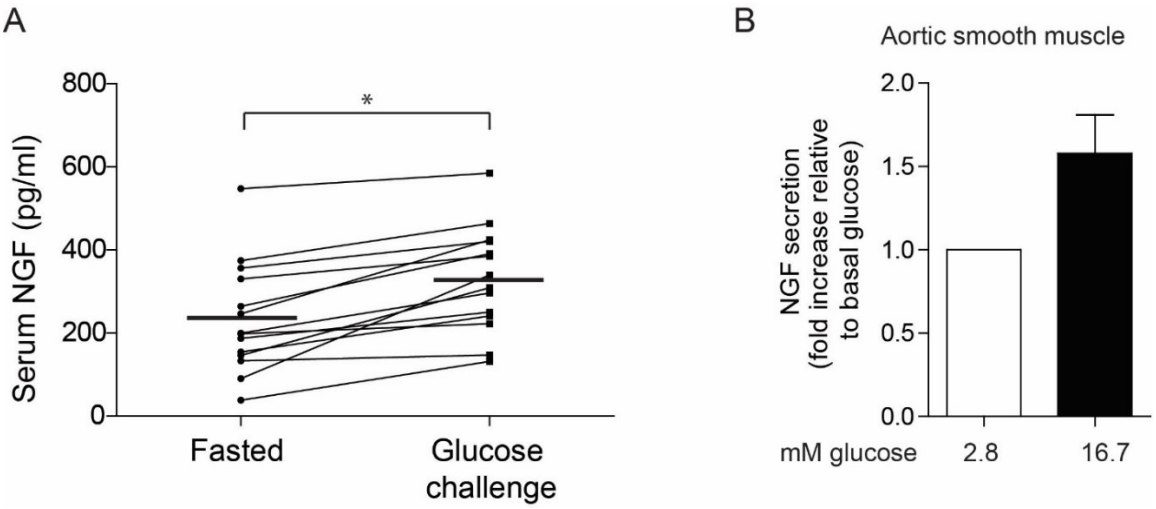


Figure 6-2: Elevated glucose stimulates acute phosphorylation of TrkA in islets

(A) High glucose stimulation (16.7 mM, 15 minutes) elicits a marked increase in phosphorylated TrkA levels in isolated islets, compared to low glucose (2.8 mM). Islet lysates were immunoblotted with a phospho-TrkA antibody, and then stripped and re-probed for TrkA for normalization. **(B)** Densitometric quantification of phospho-TrkA levels normalized to total TrkA levels. Values are the mean ± SEM from n=3 independent experiments. ∗p<0.05, *t*-test.

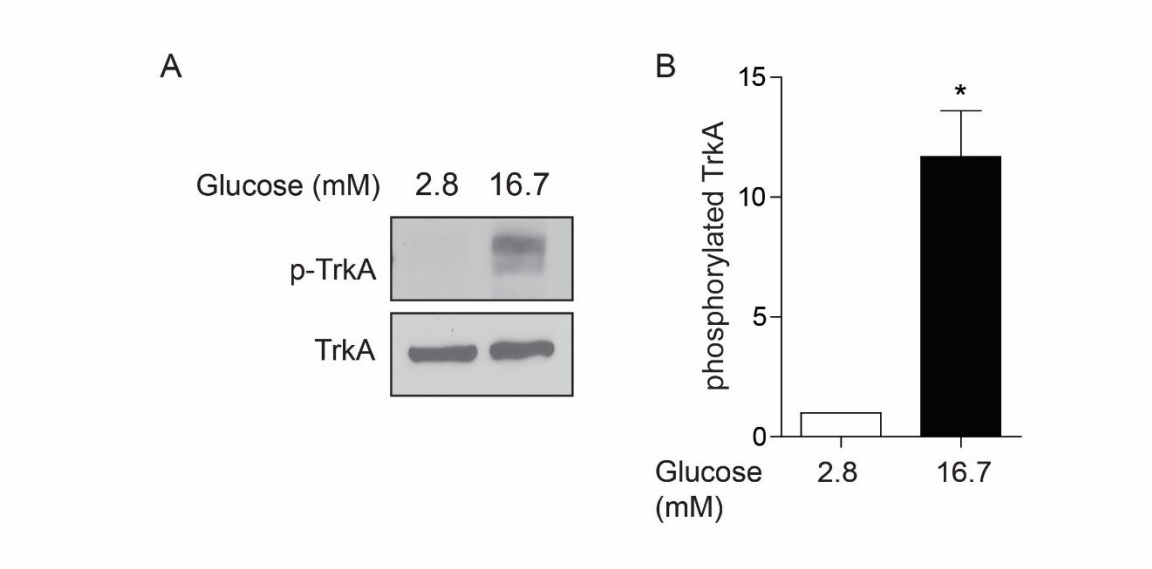
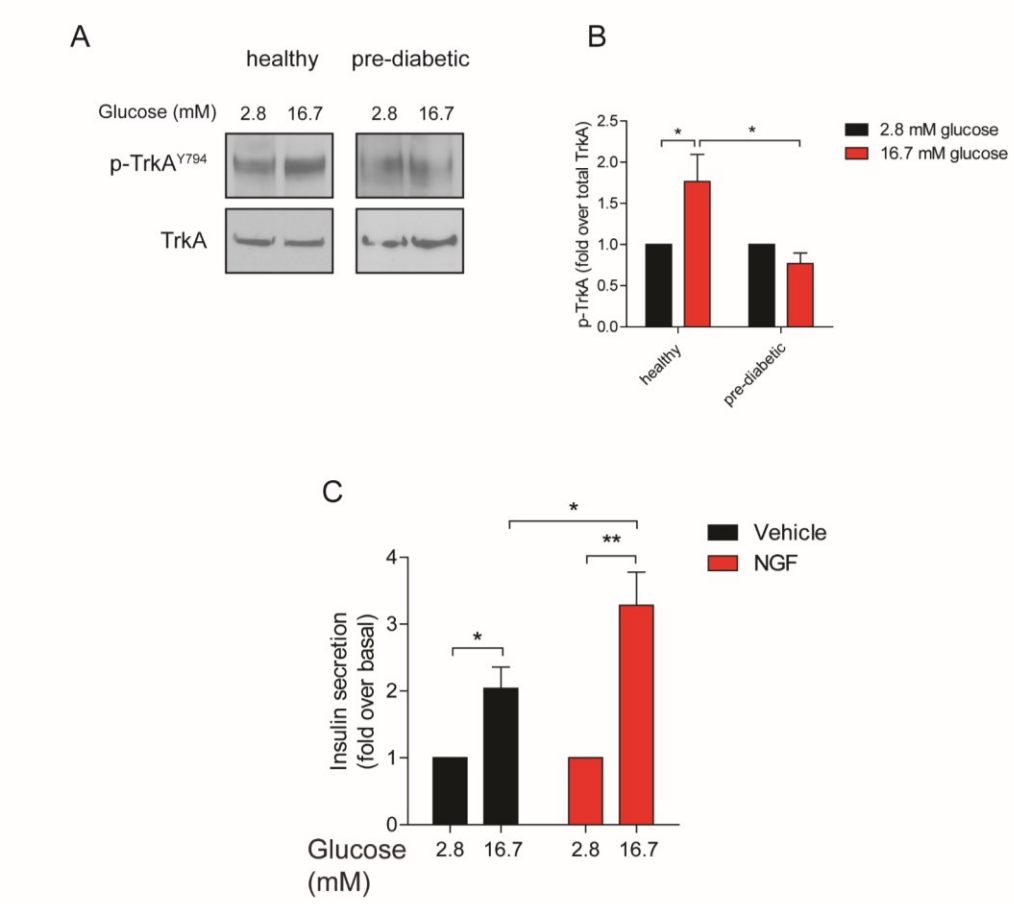


Figure 6-3: Glucose mediated activation of TrkA has implications in human islet function

(A,B) High glucose stimulation (16.7 mM, 15 minutes) induces increased phosphorylation of TrkA in healthy, but not pre-diabetic human islets. Islet lysates were immunoblotted with a phospho-TrkA antibody, and then stripped and re-probed for TrkA for normalization. Values are the mean ± SEM for n=6 non-diabetic and n=3 pre-diabetic individuals. $*p<0.05$, two-way ANOVA with Bonferroni post-test. **(C)** Islets from healthy individuals demonstrate increased GSIS in the presence of NGF (100ng/mL) (3.28 ± 0.50 fold increase over basal glucose) versus the absence (2.04 ± 0.32) n=7 individuals . $*p<0.05$, two-way ANOVA with Bonferroni post-test. Values are the mean ± SEM


A
healthy
pre-diabetic
Glucose (mM)
2.8
16.7
2.8
16.7
p-TrkA^{Y794}
TrkA
B
p-TrkA (fold over total TrkA)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
2.8 mM glucose
16.7 mM glucose
healthy
pre-diabetic
C
Insulin secretion (fold over basal)
4
3
2
1
0
*
*
**
Vehicle
NGF
Glucose (mM)
2.8
16.7
2.8
16.7

DISCUSSION

While glucose has the unique ability to trigger insulin secretion, a number of extrinsic signals enhance the secretory capacity of β-cells (Henquin et al., 2003). These extrinsic factors, including intestinal hormones and nutrients, are systemically delivered via the bloodstream to islets (Caicedo, 2013). Other than neurotransmitters derived from nerve terminals or islet cells (Caicedo, 2013), relatively little is known about factors that locally influence β-cell secretory function.

This work revealed an unexpected physiological cross-talk between nutrient and neurotrophin signaling. Elevated glucose rapidly increased NGF secretion in vivo, and profoundly enhanced TrkA phosphorylation in islets. These results argue against a merely permissive role for NGF signaling in glucose-stimulated insulin secretion, and suggest an instructive mechanism by which glucose recruits the NGF signaling axis to augment insulin secretion. This is similar to the effects of glucose on intestinal hormones and neurotransmitters that subsequently act in concert to tightly regulate a post-prandial insulin secretory response. Future studies will be of interest in elucidating the glucose sensing and signaling mechanisms that underlie glucose-mediated neurotrophin secretion in pancreatic vascular cells.

Interestingly, we provide data that highlights the relevance of neurotrophin signaling in human islets, and further suggest that this signaling may be affected in a diseased state. Our preliminary findings from 3 pre-diabetic individuals shows significantly reduced neurotrophin signaling, indicating that impaired TrkA signaling or reduced NGF secretion may be an early indicator of diabetes. In the future, it will be

important to increase our sample size of pre-diabetic individuals and perhaps investigate the state of neurotrophin signaling in overtly diabetic individuals. We would also like to determine how NGF secretion from islets is altered both in rodent models of obesity and diabetes, as well as in pre-diabetic and diabetic human islets. These investigations should greatly enhance our understanding of how NGF participates in GSIS and the progression of metabolic diseases, in particular, diabetes.

METHODS

Mice

All procedures relating to animal care and treatment conformed to Johns Hopkins University Animal Care and Use Committee (ACUC) and NIH guidelines. Mice were housed in a standard 12:12 light-dark cycle. Mice were maintained on a *C57BL/6* background, or mixed *C57BL/6* and *129P*, or *C57BL/6* and *FVB* backgrounds. Both sexes were used for analyses at 1-2 months of age, unless stated otherwise in the figure legends. $TrkA^{F592A}$ ($TrkA^{f/f}$) mice were obtained from the Jackson Laboratory (Stock No: 022362)

Human islets

We obtained human islets via the NIDDK–supported Integrated Islet Distribution Program (IIDP) from the Southern California-Islet Cell Resource Center, Scharp/Lacy Institute, University of Miami, and University of Wisconsin. Islet were obtained from 10 healthy donors (5 females, 5 males), ranging in age from 22-59 years with BMI values between 22.7 and 34.8. Islets from 3 pre-diabetic individuals (males ages 57-62, BMI 23-30.6) were also obtained. The Johns Hopkins Institutional Review Board declared the human islet material used in this study exempt from IRB review on January 7, 2016 (IRB exemption number: IRB00090529)

Antibodies

Primary antibodies for western blots include rabbit anti-Phospho-TrkA (Tyr785)/TrkB (Tyr816) (1:1000, Cell Signaling C67C8), rabbit anti-TrkA (1:1000, Millipore 06-574), and abbit anti-β-actin (1:1000, Cell Signaling, 4970).

Immunoblotting

For detection of phosphorylated TrkA, isolated islets were allowed to recover overnight in RPMI 1640 medium containing 5% fetal bovine serum (FBS) and 5 U/L penicillin/5µg/L streptomycin (for mouse islets) or CMRL 1066 medium containing 10% human serum albumin, 5 U/L penicillin/5µg/L streptomycin, 2mM GlutaMAX, and 1mM sodium pyruvate (for human islets). Islets were pre-incubated in Krebs-Ringer HEPES buffer (KRHB) containing low (2.8 mM) glucose for 1 hour, and then either left in 2.8 mM glucose or stimulated with 16.7 mM glucose in KRHB buffer for 15 minutes, and lysates prepared in boiling Laemmli buffer. Lysates were subjected to immunoblotting with rabbit anti-Phospho-TrkA (Tyr785)/TrkB (Tyr816), and membranes were later re-probed for rabbit anti-TrkA. All immunoblots were visualized with ECL Plus Detection Reagent (Thermo Scientific, 32132) and scanned with a Typhoon 9410 Variable Mode Imager (GE Healthcare). Densitometric analysis of bands was performed using ImageJ software.

***In vivo* and *in vitro* analyses of secreted NGF**

For *in vivo* NGF secretion assays, mice were fasted overnight before being injected with glucose (3 g/kg, i.p.). Blood was collected from the tail at the times indicated, spun down, and the resulting plasma fractions subjected to NGF ELISA (Millipore, CYT304). Reactions were assessed using a Tecan infinite 200 plate reader.

For i*n vitro* NGF secretion assays, primary aortic vascular smooth muscle cell cultures were established as previously described (Metz et al., 2012). Wildtype mice, 1-2 months of age, were euthanized and the aorta was dissected, de-endothelialized, and cut into small (~1mm x 1mm) squares. The squares were pressed lumen side down on a collagen-coated dish and allowed to adhere for 15-30 minutes before the addition of fresh media (DMEM media plus 20% FBS, 5 U/L penicillin/5µg/L streptomycin, 2mM GlutaMAX, and 1mM sodium pyruvate). Smooth muscle cells were allowed to migrate out of the explants and proliferate for 5 days before passaging. After the third passage in culture, smooth muscle cells were plated in 6cm dishes and allowed to grow to confluency for 3 days. Cells were equilibrated in Krebs-Ringer HEPES buffer (KRHB) containing low (2.8 mM) glucose for 40 minutes, and then 3mL of fresh Krebs-Ringer HEPES buffer with 2.8 mM glucose was added for 15 minutes and collected before the addition of 3mL Krebs-Ringer HEPES buffer with 16.7 mM glucose for 15 minutes. Supernatants (2.8 and 16.7mM glucose Krebs-Ringer HEPES buffer) were concentrated to 100µl using Amicon Ultracel 3K filter devices (Millipore) and cells were lysed with RIPA before being subjected to quantification with NGF ELISA (Millipore).

***In vitro* GSIS assays**

Human islets to recover overnight in CMRL 1066 medium containing 10% human serum albumin, 5 U/L penicillin/5µg/L streptomycin, 2mM GlutaMAX, and 1mM sodium pyruvate. Islets were washed in Krebs-Ringer HEPES buffer (KRHB) containing low (2.8 mM) glucose and allowed to stabilize for 1 hour. Groups of 5-10 islets were then

handpicked into 24-well dishes and incubated in low (2.8 mM) or high glucose (16.7 mM) in KRHB buffer with vehicle or NGF (100ng/mL) for another 30 minutes. Supernatant fractions were removed, the islets were lysed in acid ethanol, followed by insulin ELISA (ALPCO) to determine the insulin concentrations in both supernatant and islet fractions.

CONCLUSION

Portions of this chapter were previously submitted for publication at Developmental Cell: Jessica Houtz, Philip Borden, Alexis Ceasrine, and Rejji Kuruvilla (2016). *Neurotrophin signaling is required for glucose-induced insulin secretion.*

We have revealed a mechanism by which neurotrophin signaling directly influences endocrine functions. Our findings suggest a feed-forward model whereby NGF, secreted by the pancreatic vasculature in response to glucose, activates β-cell TrkA receptors to acutely promote glucose-stimulated insulin secretion **(Figure 7)**. TrkA signaling, specifically by internalized receptors, overcomes a peripheral F-actin barrier to boost insulin granule exocytosis in β-cells. Together, these findings identify a new regulatory pathway essential for insulin secretion and blood glucose homeostasis.

Neurotrophin signaling and effects outside the nervous system

To date, neurotrophins have been best-studied as essential regulators of neuronal development and neurotransmission in the vertebrate nervous system (Huang and Reichardt, 2001b). All understanding of neurotrophin signaling mechanisms has been gleaned from work in neurons. Although neurotrophins have an indisputably central role in neuronal development, the Trk family of receptors has been found to be expressed broadly outside of the nervous system. NGF and TrkA have been shown to function in several immune cell types (Aloe et al., 1997, Mazurek et al., 1986, Coppola et al., 2004, Brodie and Gelfand, 1994), the pancreas, reproductive organs (Mayerhofer et al., 1996, Li and Zhou, 2013, Jin et al., 2010), vasculature (Donovan et al., 1995, Khan et al., 2002, Nico et al., 2008), and kidneys (Shibayama and Koizumi, 1996, Sariola, 2001, Kapuscinski et al., 1996).

NGF neutralizing antibodies and the selective pharmacological inhibitor of tyrosine kinases, K252a, have allowed for some mechanistic understanding of neurotrophin signaling in non-neuronal cells types. However, there is limited *in vivo* evidence for the

specific roles of Trk receptors outside the nervous system. This deficit in data is due, in part, to the early lethality of mice with a global loss of TrkA, as well as the possible indirect consequences of loss of TrkA in the nervous system on any phenotype outside the nervous system. Our understanding of non-neuronal neurotrophin signaling would greatly benefit from in-depth, tissue-specific approaches to target Trk signaling within precise cell populations. We took advantage of recent advances in the diversity and availability of conditional mouse lines to determine where and how NGF and TrkA are functioning in the pancreas. Given the existing reports of TrkC expression in β-cells (Tazi et al., 1996) and TrkB expression in alpha-cells(Hanyu et al., 2003), similar approaches using reporter mouse lines for BDNF and NT-3 as well as conditional mouse lines for TrkB and TrkC will facilitate a better understanding of how other neurotrophins could contribute to metabolic homeostasis.

Neuronal factors in insulin secretion

Although neurons and β-cells have distinct developmental origins (Pictet et al., 1976, Fontaine and Le Douarin, 1977), they share remarkable similarities in terms of electrical properties, ion channel composition, and exocytic machinery involved in regulated secretion (Arntfield and van der Kooy, 2011). Recent reports have highlighted pleiotropic roles for well-established neurotransmitters and axon guidance factors in regulated insulin secretion. Intra-islet secretion of neuropeptide W (NPW) and pituitary adenylate cyclase-activating protein (PACAP) have both been shown to activate G-protein coupled receptors on β-cells resulting in increased [Ca2+]i and potentiated GSIS (Dezaki et al., 2008, Yada et al., 1997). Axon guidance factors including intra-islet netrin and SLIT

have been implicated in β-cell survival (Yang et al., 2011, Yang et al., 2013). In addition to β-cell survival, SLIT was also found to act through canonical ROBO receptors to regulate ER Ca2+ release and actin remodeling (Yang et al., 2013). Similarly, Rac1, a key actin regulatory protein in β-cells, was identified as a target of EphA/ephrin-A signaling in GSIS in β-cells (Konstantinova et al., 2007). These results demonstrate the importance of neuronal and paracrine factors in β-cell function. In this study, we describe a non-canonical role for neurotrophins outside of the nervous system, and also define a conserved mechanism for neurotrophin actions via signaling endosomes in non-polarized β-cells. We further demonstrated a conserved mechanism in which TrkA must undergo endocytosis and activate Rac1 in order to influence actin remodeling in response to glucose to allow for proper insulin granule secretion.

The actin cytoskeleton as a regulator of insulin secretion

The cytoskeleton is often regarded as a static structure that maintains the shape and integrity of the cell, however elements of the cytoskeleton can be quite dynamic. Microtubules and actin are key components of regulated signaling and secretory events (Gasman et al., 2004). Tubulin subunits, alpha- and beta-tubulin, comprise microtubules that form tracks along which kinesin and dynein motor proteins carry cargo such as organelles and secretory vesicles. Monomers of G-actin bind together to form filaments of F-actin upon which myosin motors move cargo and generate the tension needed for muscle contraction. The dynamic nature of “tread-milling” actin filaments is also key to the process of cell migration and endocytic events.

Microtubules have been shown to be important for the maintaining the structure of the Golgi complex that is itself a central structure in secretory vesicle maturation and movement. In the liver, microtubules are essential for lipoprotein secretion (Orci et al., 1973, Reaven and Reaven, 1980) and alcohol-induced alterations in microtubule structure and stability are thought to underlie reduced hepatic protein and membrane trafficking in alcoholism (Joseph et al., 2008). In addition to facilitating constitutive trafficking of cargo, recent work has also demonstrated a role for microtubule dynamics in insulin granule release. Hoboth et al. first demonstrated that "old" insulin granules lose their propensity to associate with microtubules and become restricted in multi-vesicular bodies destined for degradation (Hoboth et al., 2015). Zhu and colleagues further showed that microtubules in β-cells are organized into a dense meshwork rather than radial arrays extending from the cell center (Zhu et al., 2015). This dense network of microtubules restricts the mobility of insulin granules under basal conditions but, upon glucose stimulation, both microtubule depolymerization and nucleation in the Golgi result in larger "openings between filaments and increasing the availability of granules for secretion.

Although there is emerging evidence for microtubule involvement in regulating insulin secretion, a far greater body of research exists that supports a central role for actin dynamics in insulin secretion. Results have indicated both negative and positive roles for the actin cytoskeleton in secretory granule release. Early ultra-structural analysis of secretory cells indicated the presence of a cortical web of dense actin just beneath the plasma membrane that restricts granule access to the cell surface (Orci et al., 1972). F-actin dynamics have since been shown to be important for exocytosis in chromaffin cells (Vitale et al., 1995), adipocytes (Omata et al., 2000), endothelial cells (Wang et al., 2010), β-cells

(Kalwat and Thurmond, 2013), acinar cells (Valentijn et al., 1999), neurons (Morales et al., 2000), and mast cells (Wollman and Meyer, 2012). Stimulus-induced secretion can be enhanced by a number of pharmacological agents that depolymerize actin, such as cytochalasins and latrunculin toxins (Henquin et al., 2012, Li et al., 1994, Kalwat and Thurmond, 2013). Combined with structural analysis indicated granule exclusion from the cell periphery in basal conditions, these data provided strong evidence for a negative role for F-actin in secretion. However, these results must be tempered by opposing data indicating a requirement for actin filaments in sustained secretion (Kalwat and Thurmond, 2013, Uenishi et al., 2013, Varadi et al., 2005). Just as the role of microtubules in insulin secretion requires a balance between nucleation and depolymerization, actin changes in response to elevated glucose likely involve "remodeling" rather than strict deployment or stabilization during secretion.

The primary regulators of actin remodeling in β-cells are the Rho family of small GTPases, in particular Cdc42 and Rac1 (Kalwat and Thurmond, 2013, Wang et al., 2011, Nevins and Thurmond, 2003). Glucose activates Cdc42 within the first 2-3 minutes of a glucose challenge and subsequently stimulates the downstream effector, p21-associated kinase (PAK1) that is responsible for activating Rac1 (Nevins and Thurmond, 2003, Wang et al., 2011, Kalwat et al., 2013). PAK1 knockout mice are glucose intolerant and have a significantly impaired second phase of insulin secretion (Wang et al., 2011). Notably, patients with type 2 diabetes have been reported to have about an 80% reduction in PAK1 levels (Wang et al., 2011). β-cell specific loss of Rac1 or expression of dominant negative Rac1 results in similar defects in second phase insulin secretion (Asahara et al., 2013, Kowluru, 2011). Besides Rac1, PAK1 is also known to directly and indirectly activate

other actin regulators including cofilin, gelsolin, filamin A, and focal adhesion kinase (FAK) (Kalwat and Thurmond, 2013). Upon glucose stimulation, intracellular signaling through β1-integrin leads to phosphorylation of FAK and paxillin which additionally activate MAPK signaling and cytoskeletal remodeling (Cai et al., 2012, Rondas et al., 2011). Additional actin associated proteins whose activity can be modulated by glucose include N-WASP and Arp2/3, which promote actin polymerization, and scinderin that severs actin filaments in response to calcium (Uenishi et al., 2013).

β-cells have many molecular and physical properties in common with neurons, not least of which is stimulus-coupled secretion of vesicles. Once insulin granules arrive at the plasma membrane, calcium is required to for the final activation of the fusion machinery responsible for vesicle exocytosis. Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins have been extensively studied in the context of synaptic transmission, and can be generally divided into two classes, v-SNAREs that associate with vesicles, and t-SNARES that are found on the plasma membrane. In response to a stimulus, the v-SNARE, VAMP is guided to associate with the t-SNARES, syntaxin and SNAP. β-cells express several different syntaxin and SNAP isoforms which appear to function during different phases of insulin secretion (Bronk et al., 2007, Wang and Thurmond, 2009, Kalwat and Thurmond, 2013, Kalwat et al., 2012, Song et al., 2011). Syntaxin-1 and SNAP 25 are known to interact with both voltage gated calcium channels as well as potassium channels and are important for the first phase of insulin secretion (Ostenson et al., 2006). In contrast, syntaxin-4 has been implicated in regulating both first and second phase insulin secretion (Kalwat and Thurmond, 2013), and is known to bind filamentous actin and the calcium dependent actin remodeler, gelsolin (Kalwat et al., 2012). F-actin binds syntaxin-

4 at the N-terminus and may physically block incoming VAMP2 containing insulin granules from aberrantly fusing with the plasma membrane under basal glucose conditions (Jewell et al., 2008, Ostenson et al., 2006, Kalwat et al., 2012). Glucose stimulation results in dissociation of syntaxin-4 and F-actin and allows association with incoming insulin granules (Jewell et al., 2008). Additionally, syntaxin-4 interacts with gelsolin in a calcium dependent manner (Kalwat et al., 2012). Upon depolarization, and the subsequent increase in intracellular calcium, gelsolin loses its association with syntaxin-4 and becomes activated, perhaps clearing the way for insulin granule recruitment.

Although many glucose stimulated actin modulators in β-cells have been identified, the upstream molecular players that are directly activated by glucose have yet to be determined. Interestingly, there are examples of tyrosine kinase mediated actin remodeling in response to glucose. The Src family tyrosine kinase, YES was recently shown to localize to the plasma membrane in β-cells and is phosphorylated in a glucose dependent manner (Yoder et al., 2014). Additionally, EphA5/ephrin-A5 forward signaling is important for keeping insulin secretion low under basal glucose conditions, while elevated glucose triggers stimulation of reverse ephrin-A5 signaling to activate Rac1 and increase insulin secretion (Konstantinova et al., 2007).

NGF as a potential therapeutic agent for metabolic disorders

Early research investigating the influence of NGF on the pancreas revolved around its use as a potential therapy for the treatment of diabetic neuropathy (Pittenger and Vinik, 2003, Anand et al., 1996). Remarkably, NGF was one of the first growth factors to proceed to clinical trials, however issues with experimental design including variation in treatment

groups and dosage of NGF prevented further progress in its development as a viable therapeutic agent (Apfel, 2002). Additional studies *in vitro* suggest that NGF may have a direct influence on β-cell electrical potential resulting in potentiation of insulin secretion (Rosenbaum et al., 2002). Recent work has also evaluated the influence of NGF on islet transplantation (Miao et al., 2006, Hata et al., 2015). These experiments demonstrate that NGF can directly affect β-cell survival and function, leading to renewed interest in the possible pharmacological use of NGF. However there is little insight into the *in vivo* regulation of neurotrophins or the mechanisms through which they function in islets. Our work provides the first *in vivo* evidence for acute regulation of neurotrophin signaling in GSIS in the adult pancreas. In contrast to previous models suggesting autocrine NGF signaling within pancreatic β-cells, we demonstrate that NGF coming from the vasculature is acutely released to regulate actin remodeling and insulin secretion.

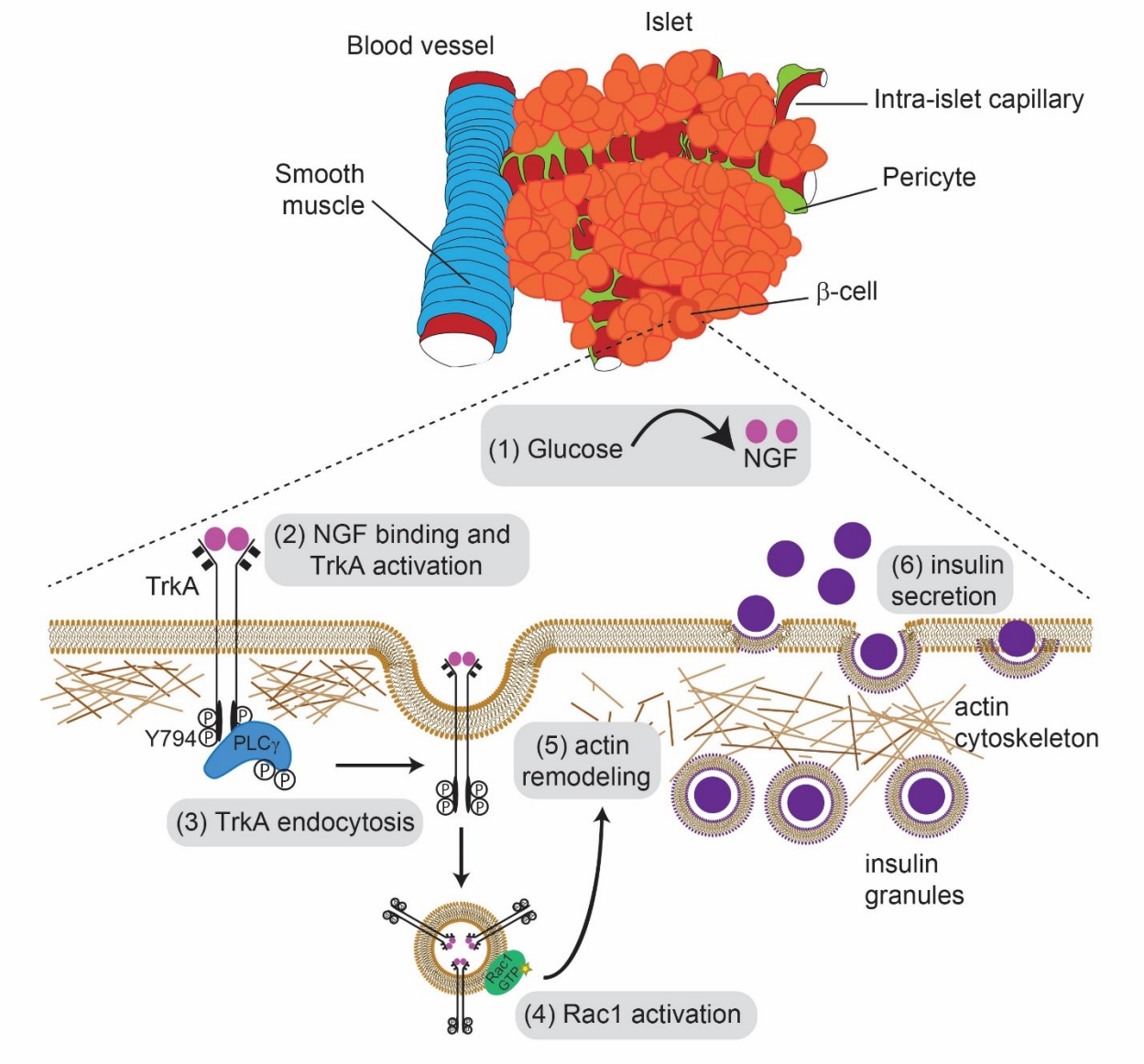
Both the discovery that NGF is supplied by the pancreatic vasculature and that circulating levels can be increased in response to glucose will be of importance for developing NGF therapies to improve β-cell function. In support of this notion, we found that TrkA is acutely phosphorylated in human islets and exogenous NGF was capable of increasing GSIS *in vitro*. Future studies determining how NGF expression and secretion are regulated in smooth muscle contractile cells will informative in deciphering how neurotrophins function differently during development and adulthood. As discussed in Chapter 4, changes in levels of NGF have been associated with several metabolic disorders (Manni et al., 2005, Civelek et al., 2013, Bullo et al., 2007), and may play an additional role in hyper-activation of the pituitary-adrenocortical axis (Otten et al., 1979) which could exacerbate hormonal imbalance leading to impaired glucose homeostasis. There are

additional parallels between stress-induced increases in NGF (Taglialatela et al., 1991) and stress related susceptibility to diabetes (Hilliard et al., 2016), which may indicate a desensitization of neurotrophin regulation of GSIS. Our results obtained from pre-diabetic human islets showed reduced activation of TrkA in response to glucose and failure to respond to NGF by potentiating insulin secretion in high glucose conditions. These findings support the idea that NGF/TrkA signaling may be an important pathway to target for metabolic screening and the development of pharmaceutical therapies.

Beyond the obvious therapeutic applications of this research to diabetes, regulation of NGF levels may be relevant to other conditions often associated with diabetes such as kidney disease and hypertension. Diabetes and hypertension are often associated and frequently occur together (Tuttle et al., 2014, Cheung and Li, 2012), and elevated glucose is known to activate the sympathetic nervous system resulting in increased blood pressure (Rowe et al., 1981). Intriguingly, vascular smooth muscle cells from hypertensive rats have increased NGF secretion (Donohue et al., 1989, Spitsbergen et al., 1995). Thus, it is tempting to speculate that the initial increase in circulating NGF reported in individuals during early stages of metabolic syndrome and diabetes (Chaldakov et al., 2003, Azar et al., 1999) may reflect a compensatory mechanism for improving insulin secretion in the face of increased metabolic demand. Indeed, contractile stimuli including arginine, vasopressin, angiotensin II, and norepinephrine can stimulate NGF secretion from smooth muscle cells (Tuttle et al., 1993, Creedon and Tuttle, 1991). In this way, alterations in NGF levels may be prognostic of a susceptibility to develop certain complications associated with diabetes and obesity.

Figure 7. Neurotrophin signaling acutely promotes glucose-induced insulin secretion via actin reorganization in β-cells

(1) NGF is secreted by pancreatic vascular smooth muscle cells and intra-islet pericytes in response to elevated glucose. (2) Vascular-derived NGF activates TrkA receptors on islet β-cells. (3) TrkA phosphorylation on Y794 leads to association and activation of the downstream effector, PLCγ, which triggers receptor internalization. (4) Endosomal signaling from internalized TrkA receptors recruits and activates the actin modulatory protein Rac1, to (5) remodel a peripheral F-actin barrier, and (6) promote insulin granule exocytosis.


Islet
Blood vessel
Intra-islet capillary
Smooth muscle
Pericyte
β-cell
(1) Glucose
NGF
(2) NGF binding and TrkA activation
TrkA
(6) insulin secretion
Y794
PLCγ
(5) actin remodeling
actin cytoskeleton
(3) TrkA endocytosis
insulin granules
Rac1 GTP
(4) Rac1 activation

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CURRICULUM VITAE

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BIRTH

February 12, 1988 in Lexington, KY.

EDUCATION

Johns Hopkins University, Baltimore, MD, Graduate program in Cell, Molecular, Developmental Biology, and Biophysics (CMDB)
Ph.D in Biology
Anticipated July 2016

University of Kentucky, Lexington, KY
Bachelor of Science, Agricultural Biotechnology
May 2010
Minor: Biology, Chemistry, and Mathematics
GPA: 3.8
Summa Cum Laude
Honors Program, Dean's List
Phi Kappa Phi Honor Society member (2009)

RESEARCH EXPERIENCE

PH.D. STUDENT
2010-current
Laboratory of Rejji Kuruvilla
Department of Biology, Johns Hopkins University, Baltimore, MD
Research project: "Neurotrophin signaling is required for glucose induced insulin secretion"

BECKMAN SCHOLAR
2008-2010
Laboratory of Karyn Esser

Department of Physiology, University of Kentucky, Lexington, KY

Research project: "Characterization and Computational Modeling of Sarcomere Structure in the Muscle Specific Bmal1 knock-out Mouse"

AMSTEMM RESEARCH FELLOWSHIP
2008
Laboratory of Karyn Esser
Department of Physiology, University of Kentucky, Lexington, KY
Research project: "Circadian Regulation of microRNA-206 in Skeletal Muscle"

UNDERGRADUATE RESEARCH ASSISTANT
2008
Laboratory of James MacLeod
Department of Veterinary Science, University of Kentucky, Lexington, KY
Collaborated with a graduate student on an axolotl (*Ambystoma mexicanum)* cartilage regeneration/repair project.

UNDERGRADUATE RESEARCH ASSISTANT
2007
Laboratory of Guiliang Tang
Department of Plant and Soil Sciences, University of Kentucky, Lexington, KY
Collaborated with lab members on current research projects involving microRNAs in *Arabidopsis thaliana*, *Nicotiana tabacum,* and *Solanum lycopersicum*. Aided in general lab maintenance.

DATA COLLECTION ASSISTANT
2006-2009
Equine Analysis Systems Inc., Lexington, KY (Fasig Tipton and Keeneland Sales)

Aided in the collection of ultrasound heart scans on yearling and brood mares

GRANTS and AWARDS

Integrated Islet Distribution Program (IIDP) Pilot grant
2016
"Neurotrophins as New Regulators of Islet Biology"

Contributed to: National Institutes of Health NIDDK R01: DK108267
2016

Poster Prize
2014

CMDB Graduate Program JHU Retreat

University Kentucky, College of Agriculture, Outstanding Senior Award
2009

Beckman Scholar Award
2009

AMSTEMM Research Fellowship
2009

PUBLICATIONS

Houtz J, Borden P, Ceasrine A, Kuruvilla R. Neurotrophin signaling is required for glucose induced insulin secretion. *In review at Developmental Cell*.

Houtz J, Kuruvilla R. (2014). VIP pipes up: neuronal signals direct tubulogenesis. Developmental Cell. 30(4):361-2. doi: 10.1016/j.devcel.2014.08.011

Borden P, **Houtz J**, Leach SD, Kuruvilla, R. (2013). Sympathetic innervation during development is necessary for pancreatic islet architecture and functional maturation. Cell Reports. 4(2):287-301. doi: 10.1016/j.celrep.2013.06.019.

POSTER PRESENTATIONS

Gordon Research Conference: Neurotrophic Factors (June 2015)
Jessica Houtz, Philip Borden, Mehboob Hussain, Rejji Kuruvilla
"An essential role for neurorophin signaling in insulin secretion"

Keystone Symposium of Emerging Concepts and Targets in Islet Biology (April 2014)
Jessica Houtz, Philip Borden, and Rejji Kuruvilla
"Neurotrophin Signaling and Functions in the Pancreas"

Experimental Biology Meeting (2010)
Jessica I. Houtz, P. Christopher Hatfield, Kenneth S. Campbell, Karyn A. Esser
"Muscle specific influences of Bmal1 on myofilament interactions"

Biophysical Society Conference (2010)
Jessica I. Houtz, Patrick C. Hatfield, Kenneth S. Campbell, Karyn A. Esser
"Automated Image Analysis of Electron Micrographs of Structurally Compromised Striated Muscle"

Gill Heart 12th Annual Cardiovascular Research Day
Jessica I. Houtz, Patrick C. Hatfield, Kenneth S. Campbell., Karyn A. Esser (2009) "Automated Image Analysis of Electron Micrographs of Structurally Compromised Striated Muscle"

University of Kentucky Center for Muscle Biology Retreat
Jessica I. Houtz, Patrick C. Hatfield, Kenneth S. Campbell., Karyn A. Esser (2009) "Automated Image Analysis of Electron Micrographs of Structurally Compromised Striated Muscle"

TEACHING AND MENTORING EXPERIENCE

TEACHING ASSISTANT, Department of Biology, JHU
Genetics lab (Fall 2011)
Genetics (Fall 2012, 2013)
Developmental Biology lab (Spring 2012)
Cell Biology (Spring 2013, 2014)

LABORATORY MENTOR, Kuruvilla Lab, Department of Biology, JHU
Lauren Lipshutz (Undergraduate student, Summer 2013)
Nathan Snyder (Graduate rotation student, Fall 2013)
Muhammad Hamza (Undergraduate student, Summer 2014)
Alexis Ceasrine (Graduate rotation student, Winter 2014)

PEER MENTOR, College of Agriculture, University of Kentucky.
Fall 2008, Acted as student mentor in a general agriculture class required for all incoming freshman to the College of Agriculture.

- Developed class discussions
- Aided in teaching and lesson planning
- Evaluation of student performance
- Involved students in College of Agriculture activities

LEADERSHIP EXPERIENCE

PRESIDENT
2009-2010
Agricultural Biotechnology Club, University of Kentucky
Organized club meetings, applied for student government grants, recruited speakers, coordinated club events with Ag. Biotech faculty and staff

RECRUITMENT CHAIR
2007-2009

Agricultural Biotechnology Club, University of Kentucky
Planned club meetings, advertised meeting dates, engaged incoming freshmen in the club participation.